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Intestinal anti-inflammatory activity of probiotics in experimental models of colitis: impact of cell viability and role of miRNAs.

Tesis Doctoral

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2015

Editorial: Universidad de Granada. Tesis Doctorales
Autora: Alba Rodríguez Nogales
ISBN: 978-84-9125-283-2
URI: http://hdl.handle.net/10481/40663

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Intestinal anti-inflammatory activity of probiotics in experimental models of colitis: impact of cell viability and role of miRNAs.

Tesis doctoral para aspirar al grado de doctor presentada por

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Bajo la dirección de los doctores

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Granada, 2015

Universidad de Granada

Departamento de Farmacología

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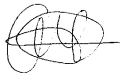

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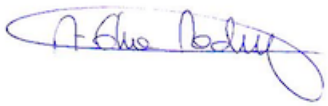

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A mi familia…..

….Los imposibles de hoy serán posibles mañana….
Konstantin Tsiolkovsky

….No dejes apagar el entusiasmo, virtud tan valiosa como necesaria; trabaja, aspira, tiende siempre hacia la altura….
Ruben Darío

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1. Introducción

El término Enfermedad Inflamatoria Intestinal (EII) comprende dos patologías: la enfermedad de Crohn y la colitis ulcerosa. Ambos cuadros clínicos se caracterizan por una inflamación crónica y recurrente del tracto gastrointestinal, de etiología desconocida, en la que se alternan periodos de exacerbación de los síntomas, seguidos de intervalos más o menos prolongados de remisión de los mismos (Baumgart & Sandborn, 2007; Fiocchi, 1998).

Aunque hasta el momento se desconocen los mecanismos responsables de la iniciación y perpetuación en el tiempo del proceso inflamatorio intestinal, es aceptado que en su fisiopatología están implicados factores genéticos, ambientales e inmunológicos (Figura 1). Así, numerosos estudios han propuesto que, en personas genéticamente predispuestas, una activación exagerada y descontrolada del sistema inmune intestinal frente a un determinante antigénico desconocido, puede desencadenar la aparición de la respuesta inflamatoria intestinal exacerbada (Podolsky, 2002). Esta respuesta inmunológica genera numerosos mediadores de carácter pro-inflamatorio (citocinas, eicosanoides y metabolitos reactivos derivados del oxígeno o del nitrógeno) que actúan de forma sinérgica y simultánea promoviendo la amplificación y cronificación del proceso inflamatorio intestinal (Abraham & Cho, 2009; Baugh et al., 1999; Boughton-Smith, 1994; Chin & Parkos, 2006; Sartor, 1997).

Asimismo, son numerosos los estudios que sugieren que la microbiota juega un papel clave en el inicio y desarrollo de la EII (Guarner et al., 2002). Se propone que existe un desequilibrio en las concentraciones luminales de determinadas bacterias en pacientes con EII, proceso llamado disbiosis, de forma que el incremento en la proporción de bacterias potencialmente agresivas, como *Bacteroides*, cepas adhesivas/invasivas de *Escherichia coli*, y enterococos, y la disminución de poblaciones protectoras como lactobacilos y bifidobacterias (Darfeuille-Michaud et al., 2004; Farrell & LaMont, 2002; Neut et al., 2002).

El papel que la microbiota puede desempeñar en la patogénesis de la EII ha sido reforzado por numerosas evidencias y observaciones llevadas a cabo en los últimos años. Se ha observado un incremento en el número de bacterias entéricas y

sus metabolitos en la mucosa inflamada de sujetos aquejados de EII (Guarner et al., 2002; Schultsz, Van Den Berg, Ten Kate, Tytgat, & Dankert, 1999; Swidsinski, Weber, Loening-Baucke, Hale, & Lochs, 2005). Relacionado con esto, se ha observado que las zonas más frecuentemente afectadas por el proceso inflamatorio en la EII (íleon distal y colon) son las regiones del intestino con mayor carga bacteriana (Sartor, 1997). La composición de la microbiota intestinal se encuentra alterada en estos pacientes. La evidencia más contundente, deriva de modelos experimentales que muestran como el proceso inflamatorio intestinal que se desarrolla de forma espontánea en distintos animales transgénicos, no tiene lugar cuando los animales son mantenidos en condiciones libres de gérmenes (Rath et al., 1996; Sadlack et al., 1993; Taurog et al., 1994).

Por otra parte, recientemente, se ha puesto de manifiesto el papel de los micro-RNAs (miRNA) en diferentes patologías. Los miRNAs son pequeñas moléculas endógenas de ácido ribonucleico (RNA) no codificante implicados en numerosos procesos biológicos (O'Connell, Rao, & Baltimore, 2012), entre los que se incluyen la regulación del sistema inmune; debido a esto se les relaciona con la patogénesis de numerosas enfermedades, como la EII (Biton et al., 2011; Paraskevi et al., 2012; Pekow et al., 2012; Wu et al., 2008). Por otro lado, han surgido determinadas evidencias del papel de la microbiota intestinal en la regulación de la expresión de los miARNs (Dalmasso et al., 2011; Xue et al., 2011). Evidencias, sustentadas en estudios realizados en diferentes modelos de animales y en humanos, en los que se ha visto una asociación entre la modificación de la expresión de determinados miARNs y la composición y diversidad de la microbiota intestinal (Feng et al., 2012; Singh et al., 2012; Xue et al., 2011).

Por lo tanto, una estrategia terapéutica potencialmente útil en el control de estas enfermedades consistiría en restablecer el equilibrio en la microbiota del lumen intestinal, lo que podría obtenerse mediante la administración de determinados probióticos (Gionchetti et al., 2006).

En base a esto, la evidencia más significativa del uso de probióticos en la EII, es aquella procedente de ensayos clínicos en los que se ha evaluado la efectividad del probiótico *Escherichia coli* Nissle 1917 o de la mezcla de probióticos VSL#3 y que muestran su utilidad como tratamientos para el mantenimiento del estado de remisión y la prevención de las recaídas (Schultz & Lindstrom, 2008)(Scott & Aberra, 2011).

Los mecanismos propuestos como responsables de los beneficios derivados de su uso en la EII incluyen: la producción de compuestos antibacterianos y la reducción del pH, acciones por las que modifican la composición de la microbiota intestinal, inhibiendo el crecimiento de bacterias nocivas; su capacidad de desplazar a estas bacterias de su sitio de unión al epitelio; la mejora de la función de barrera intestinal; y la modulación de la respuesta inmune de la mucosa del hospedador (Rioux & Fedorak, 2006) (Figura 2). Sin embargo, no todos los probióticos comparten las mismas propiedades, cada uno posee mecanismos de acción individuales, y es el estado del hospedador el que va a condicionar la elección de la especie o cepa optima (Shibolet et al., 2002).

Por lo tanto, teniendo en cuenta la etiología de la EII, y que actualmente no existe un tratamiento adecuado que combine eficacia y ausencia de efectos adversos; sería interesante desarrollar estrategias terapéuticas que corrijan la disbiosis, y que al mismo tiempo, controlen la respuesta inmune alterada que promueve el proceso inflamatorio intestinal.

Así que, se propusieron dos objetivos principales:

1) Establecer una posible relación entre la modificación de la microbiota intestinal, el perfil de la expresión de miRNAs y el desarrollo del proceso inflamatorio en dos modelos de colitis experimental.

2) Evaluar si el efecto anti-inflamatorio mostrado por los diferentes probióticos está relacionado con la modificación de la microbiota intestinal; y si, la modulación de la respuesta inmune intestinal puede estar asociada con una modificación en la expresión de aquellos miRNAs que se hayan visto alterados en el proceso inflamatorio.

Por otro lado; aunque tradicionalmente, la viabilidad del probiótico ha sido requisito imprescindible para ejercer efectos beneficiosos en la EII; han aparecido, recientemente, estudios que revelan que determinados probióticos podrían ejercer efectos anti-inflamatorios en diferentes modelos experimentales de colitis sin que la viabilidad sea condición indispensable. En este contexto, en esta tesis se evaluará si la viabilidad de uno de los probióticos, *Lactobacillus fermentum* CECT5716, es esencial para ejercer su actividad en estas condiciones intestinales.

Para llevar a cabo nuestros objetivos se usaron 4 cepas de probióticos diferentes: *Lactobacillus fermentum* CECT5716, *Lactobacillus salivarius* CECT5713, *Escherichia coli* Nissle 1917 y *Saccharomyces boulardii* CNCMI-745. Los probióticos fueron administrados de forma oral y como tratamiento preventivo en los dos modelos de colitis experimental, en el modelo del sulfato de dextrano sódico (DSS) (Dextran Sulfate Sodium) y en el del ácido sulfónico dinitrobenceno (DNBS) (Dinitrobenzene Sulfonic Acid) en ratones (Figuras 5 y 6). Durante el desarrollo de las experiencias se controlaron diariamente una serie de parámetros generales, que incluyen el consumo diario de comida de los animales, la evolución del peso corporal, y la aparición de heces diarreicas y sanguinolentas por visualización de restos perianales (Bell, Gall, & Wallace, 1995).

Tras el sacrificio de los animales, el colon fue extraído en su totalidad. El tejido colónico fue fragmentado para determinaciones bioquímicas y extracción de RNA y miRNAs. Además, se recogieron los contenidos fecales para la extracción de ácido desoxirribonucleico (DNA) bacteriano genómico en su totalidad y se realizó la amplificación del gen 16S del ARNr, concretamente la región V1-V3. Posteriormente, se llevó a cabo la secuenciación del material genético por pirosecuenciacion de los amplicones (Zhang, Luo, Fang, & Wang, 2010). Las alteraciones intestinales fueron caracterizadas en base a parámetros macroscópicos y bioquímicos, evaluando el efecto antiinflamatorio intestinal de los diferentes tratamientos administrados.

De forma adicional, los diferentes efectos inmunomoduladores directos por parte de los diferentes probióticos se comprobó *in vitro* sobre la línea epitelial de mucosa colónica (CMT-93) y en macrófagos derivados de la medula ósea (BMDM), dos tipos celulares implicados en la respuesta inmune intestinal.

Finalmente, para evaluar si es requisito indispensable la viabilidad del probiótico, *Lactobacillus fermentum* CECT5716, en el efecto anti-inflamatorio intestinal se llevaron a cabo estudios *in vivo* e *in vitro*. Para ello, se utilizó el modelo del ácido trinitrobencenosulfónico (TNBS) en ratas. La muerte del probiótico se llevó a cabo sometiéndolo a shock térmico durante 30 minutos a 95ºC. El probiótico, tanto vivo como muerto, fue administrado oralmente durante dos semanas antes de la inducción de la colitis con TNBS y se continuó hasta completar el ensayo. Después de tres semanas, los animales fueron sacrificados y el daño macroscópico fue valorado en función de la relación peso/longitud del colon. En el modelo del TNBS se asignó el daño macroscópico (IDM) de acuerdo con el criterio descrito por Bell y col. (1995) (Bell et al., 1995) (Tabla3). Las determinaciones bioquímicas incluyeron la valoración

del estado oxidativo colónico, mediante la determinación de la actividad del enzima mieloperoxidasa (MPO) (Krawisz, Sharon, & Stenson, 1984), el contenido de glutation (GSH) total (Anderson, 1985) y la expresión proteica del enzima óxido nítrico sintasa inducible (iNOS) intestinal mediante Western blot (Camuesco et al., 2004). Asimismo, se procedió al análisis de la expresión génica, mediante reacción en cadena de la polimerasa (PCR) de forma cuantitativa, de distintos marcadores del proceso inflamatorio, como factor de necrosis tumoral (TNF)-α e interleucinas(IL)-1β.

Finalmente, se evaluó el efecto de la viabilidad del probiótico *in vitro*, en dos líneas celulares implicadas en la respuesta inmune, la línea Caco-2 de adenocarcinoma humano y las RAW 264.7, macrófagos murinos. En células Caco-2, se determinó IL-8 mediante técnicas de *Enzyme-Linked ImmunoSorbent Assay* (ELISA), mientras que la expresión proteína de p44/42 y p38 (ruta de señalización de las MAP quinasas) fue determinada por Western blot. En células RAW 264.7 se determinó el efecto del probiótico, tanto vivo como muerto, con el uso de la técnica ELISA, con la que se midió la producción de IL-1β; y los niveles de nitritos producidos por el método de Griess (Green et al., 1982).

2. Resultados

En el modelo del DSS en ratones, en el que se administraron los probióticos de forma preventiva durante 26 días, periodo tras el cual los ratones tratados experimentaron una mayor recuperación del daño intestinal (disminución del índice de actividad de la enfermedad, menor relación peso/longitud del colon), asociada a una reducción en la producción de citocinas pro-inflamatorias como IL-1β (Figura 9.A), una disminución de la expresión de enzimas como *matrix metalloproteinase* (MMP)-2, MMP-9 e iNOS. (Figura 9.B). Por otra parte aumentaron la expresión de proteínas de integridad de la membrana epitelial como mucinas(MUC)-2, MUC-3, occludina(OCLN) y *zonula occludens* (ZO)-1 (Figura 9.B). Al comprobar los miRNAs alterados en ambos modelos DSS y DNBS, pudimos observar que en modelo del DSS, los ratones colíticos presentaban una expresión más elevada con respecto al sano de miR-10, miR-155 y miR-223; y una reducción de la expresión miR-143 y miR-375 (Figura 10). Asimismo, el tratamiento con los diferentes probióticos resultó, de forma general, que los probióticos modifican la expresión de la mayoría de los miRNAs, aunque su actuación difiere entre ellos. Todos los probióticos mostraron efectos significativos en la regulación de miR-155 y miR-223 (Figura 10); y, solo *L.fermentum* y *E.coli* Nissle

1917 mostraron efecto en la regulación de la expresión de miR-143 y miR-150 (Figura 10). Para evaluar la composición de la microbiota y su posible alteración en modelo de colitis y con los diferentes tratamientos realizamos la secuenciación del DNA extraído de las muestras fecales; y calculamos diferentes índices ecológicos como; índice de Shannon (parámetro que combina riqueza y uniformidad); de Pielou (muestra la presencia eventual de algún individuo además de cómo se distribuye en la muestra) y por último el índice de Chao (índice de riqueza estimada). Además, se calculó la abundancia de los principales filos bacterianos y la ratio de los dos mayoritarios, *Firmicutes* y *Bacteriodetes*. La relación *Firmicutes* y *Bacteriodetes,* conocida como F/B, es un potencial marcador para evaluar una situación de disbiosis y/o patológica (Mariat et al., 2009; Sanz & Moya-Perez, 2014; Youmans et al., 2015). Los resultados obtenidos revelaron que solo la administración de *L.fermentum* y *E.coli* Nissle 1917 fue capaz de incrementar el valor de los tres parámetros ecológicos, que se vio disminuido en los ratones colíticos (Figura 11). Por el contrario, el ratio F/B sufrió un drástico incremento comparado con el grupo de animales sanos (Figura 12). Este incremento se relacionó con una alta abundancia de Firmicutes y una reducción de abundancia de Bacteriodetes; resultado que se relaciona con estudios previos en condiciones de inflamación intestinal en humanos (Frank et al., 2007; Jeffery et al., 2012; Krogius-Kurikka, et al., 2009; Krogius-Kurikka, Lyra, et al., 2009). En este caso todos los tratamientos mostraron efecto restaurando los valores con la excepción de *S.boulardii* (Figura 12).

Una vez evaluados los efectos de los diferentes probióticos en el modelo del DSS, de la misma forma procedimos a evaluarlos en el modelo del DNBS en ratón. El tratamiento de los probióticos tuvo una duración total de 24 días. La inducción de la colitis con DNBS fue realizada al día 20 de tratamiento. La colitis experimental inducida fue caracterizada por una reducción progresiva de peso, asociada a reducida ingesta de comida y presencia de diarrea (Figura 13). Aunque no se observó diferencias significativas en las medidas anteriores por parte de los tratamientos, una vez sacrificados los animales se procedió a la determinación bioquímica y consiguiente evaluación de la microbiota. Si bien, no se observaron diferencias macroscópicas, si pudimos observar cómo el tratamiento con los probióticos *L.fermentum* y *E.coli* Nissle 1917 fue capaz de disminuir la expresión de citocinas pro-inflamatorias como IL-1β y TNF-α, además de MMP-2 (Figura 14). Además, todos los probióticos restauraron de forma significativa la expresión de proteínas de integridad de la membrana epitelial como MUC-3 y OCLN (Figura 14). El proceso inflamatorio en el modelo del DNBS fue asociado con modificaciones en la expresión de miRNAs. El

grupo colítico presentó un incremento en la expresión de miR-155 y miR-223 y una reducción de miR-143, miR-150 y miR-375. Los tratamientos con los diferentes probióticos no mostraron el mismo perfil con respecto a estos miRNAs, con la excepción del miR-150 que fue restaurado de forma significativa por todos los probióticos (Figura 15). Cuando la microbiota fue evaluada, los análisis de los diferentes parámetros ecológicos, mostraron que en este caso, los animales colíticos sólo presentaron una disminución de la diversidad microbiana, como puso de manifiesto la reducción del valor del índice de Shannon (Figura 16). Esta disminución fue restaurada por todos los probióticos excepto por *S.boulardii* (Figura 16). El cálculo del ratio F/B, en el modelo del DNBS, fue disminuido de forma significativa con respecto al grupo sano y restaurado sólo por los tratamientos con *L.fermentum* y *E.coli* Nissle (Figura 17). Esta disminución del ratio y posterior restauración por parte de los probióticos se relacionó con una disminución de la abundancia del filo *Firmicutes* (Figura 17).

Para llevar a cabo una mejor caracterización de las propiedades inmunomoduladoras de los probióticos y examinar su papel en el efecto anti-inflamatorio intestinal mostrado se realizaron estudios *in vitro* en células CMT-93 y BMDM, determinando la expresión de diferentes marcadores implicados en la respuesta inflamatoria. En CMT-93 todos los probióticos mostraron un incremento en la expresión de MUC-3; solo *E.coli* Nissle fue capaz de disminuir la expresión de TNF-α y finalmente, todos presentaron una reducción de la expresión de IL-6 con excepción de *L.salivarius* (Figura 18). Sin embargo, en BMDM, *L.fermentum* fue el único tratamiento que mostró una mejora de la expresión de TNF-α, iNOS e IL-6 (Figura 19).

Finalmente, la evaluación de la viabilidad del probiótico, *L.fermentum,* como requisito indispensable para ejercer efecto beneficioso, mostró *in vivo* que el pre-tratamiento con el probiótico, tanto vivo como muerto, redujo el daño inducido por TNBS comparado con los animales colíticos. Así, el efecto anti-inflamatorio fue demostrado por una reducción significativa de los valores del índice de daño macroscópico (Figura 20). Los grupos tratados con el probiótico en las diferentes condiciones, mostraron una reducción en la actividad MPO, un buen marcador de la infiltración granulocitica (Krawisz et al., 1984) que se encuentra incrementado como consecuencia del proceso inflamatorio. El estrés oxidativo fue contrarrestado igualmente por el tratamiento con el probiótico en ambas condiciones como muestra la parcial recuperación de los niveles de glutatión total, uno de los principales

compuestos implicados en la respuesta antioxidante fisiológica (Grisham, 2002) que resultó disminuido en los animales colíticos sin tratamiento (Figura 21). Igualmente, ambas condiciones del probiótico disminuyeron TNF-α e IL-1β, dos de las principales citocinas involucradas en el proceso inflamatorio (Macdonald & Monteleone, 2005; Strober & Fuss, 2011); además de reducir significativamente la expresión de la enzima iNOS, principal producto de *nitric oxide* (NO), mediador pro-inflamatorio que juega un papel clave en la patogénesis de la EII (Figura 21) (Zingarelli, Szabo, & Salzman, 1999). También, se realizaron estudios *in vitro* en dos tipos celulares implicados en la respuesta inmune, células Caco-2 y RAW 264.7. Los resultados de la incubación del probiótico, vivo y muerto, en ambas líneas celulares resultó en una reducción de la producción de mediadores inflamatorios producidos por estas células tras ser estimuladas. Concretamente, ambas formas del probiótico, en células Caco-2, han reducido la producción de los niveles de IL-8 y de la expresión de las proteínas, p44/42 y p38 de la vía de las MAP kinasas (Figura 23). Asimismo, en células RAW 264.7, la incubación de ambas condiciones del probiótico disminuyó los niveles de IL-1β y nitritos. (Figura 24).

3. Conclusiones

1. La administración de los probióticos como tratamiento preventivo ejerce un efecto anti-inflamatorio intestinal tanto en el modelo experimental de DSS como en el del DNBS en ratón. Su habilidad para modificar la composición de la microbiota y las propiedades inmunomoduladoras características de estas bacterias están implicadas en sus efectos beneficiosos. El tratamiento de los probióticos muestra un impacto positivo en la respuesta inmune innata y adaptativa; además, de conseguir modificar a nivel post-transcripcional diferentes miRNAs, los cuales se han visto alterados en condiciones de estado inflamatorio intestinal.

2. Todos los probióticos evaluados, al igual que otros usados en la terapia de la EII, ejercen un efecto inmunomodulador, evidenciado por su efecto *in vitro* en células epiteliales colónicas y macrófagos, en los cuales consiguieron restaurar la expresión de diferentes marcadores implicados en la respuesta inmune.

3. Los mecanismos implicados en los efectos anti-inflamatorios parecen ser dependientes del probiótico utilizado, ya que cada probiótico ha presentado un patrón diferente en la modulación de la expresión de los diferentes marcadores inflamatorios evaluados, así como en la modificación de la composición de la microbiota.

4. La viabilidad del probiótico *L.fermentum* CECT5716 no es esencial para ejercer su efecto anti-inflamatorio. Ambas condiciones, vivo y muerto, han demostrado atenuar el proceso inflamatorio en el modelo del TNBS así como *in vitro*. Esto podría proporcionar un valor añadido al probiótico e implicaría la revisión del generalmente aceptado concepto de probiótico.

Introduction

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a chronic gastrointestinal inflammation characterized by alternating relapses and remissions that comprises two major conditions: Crohn's disease (CD) and ulcerative colitis (UC). Both forms of IBD are featured by exacerbated uncontrolled intestinal inflammation that leads to poor quality of life and requires prolonged medical and/or surgical interventions.

Histologically, examination of intestinal tissue from patients with active disease shows inflammatory cell infiltration corresponding with dramatic tissue injury including oedema, loss of goblet cells, fibrosis, erosions and ulcers. The inflammation associated with CD can discontinuously affect any part of the gastrointestinal tract, from the mouth to the anus but it is usually, although not always, localized in the distal small bowel and/or colon. The inflamed bowel obtained from patients with active CD reveals transmural inflammation characterized by the presence of large numbers of acute and chronic inflammatory cells within the mucosa, submucosa and muscularis propia (Baumgart & Sandborn, 2007; Kang et al., 2008). The clinical presentation is largely dependent on disease location and can include diarrhoea, abdominal pain, fever, clinical signs of bowel obstruction, as well as passage of blood or mucus or both (Baumgart & Sandborn, 2007). On the other hand, UC is characterized by a non-transmural inflammation, which is restricted, exclusively, to the rectum and large bowel (Vucelic, 2009). Typically, the inflammatory changes are limited to the mucosa and submucosa with cryptitis and crypt abscesses, and the inflammatory cell composition is similar to CD. Patients usually present bloody diarrhoea, passage of pus, mucus, or both, and abdominal cramping during bowel movements (Baumgart & Sandborn, 2007).

Etiopathogenesis

The aetiology of IBD is not fully understood (Kaser, Zeissig, & Blumberg, 2010). In fact, for at least two decades, IBD has been the focus of intense attention at the basic science, translational and clinical level, which resulted in an exponential growth in the knowledge of its putative predisposing factors, possible cause(s), and underlying cellular and molecular mechanisms. This indisputable progress resulted in an improved understanding of IBD pathogenesis and the characterization of new

biomarkers, thus providing more precise diagnostic tools, as well as the development of novel therapies.

Many theories have been proposed to explain IBD pathogenesis, ranging from infectious to psychosomatic, social, metabolic, vascular, genetic, allergic, autoimmune and immune-mediated mechanisms (Fiocchi, 2013; Kaser, et al., 2010; Xavier & Podolsky, 2007). Currently, there is a general agreement that IBD is the result of the combined effects of four basic components (Figure 1): multiple genetic variations, alterations in the composition of the intestinal microbiota, changes in the surrounding environment and the reactivity of the intestinal mucosal immune response. There is also a general consensus on the conclusion that none of these four components can by itself trigger or maintain the intestinal inflammation but it is their integration and reciprocal influence what determines whether IBD will appear and with which clinical phenotype (Triantafillidis, Merikas, & Georgopoulos, 2011).

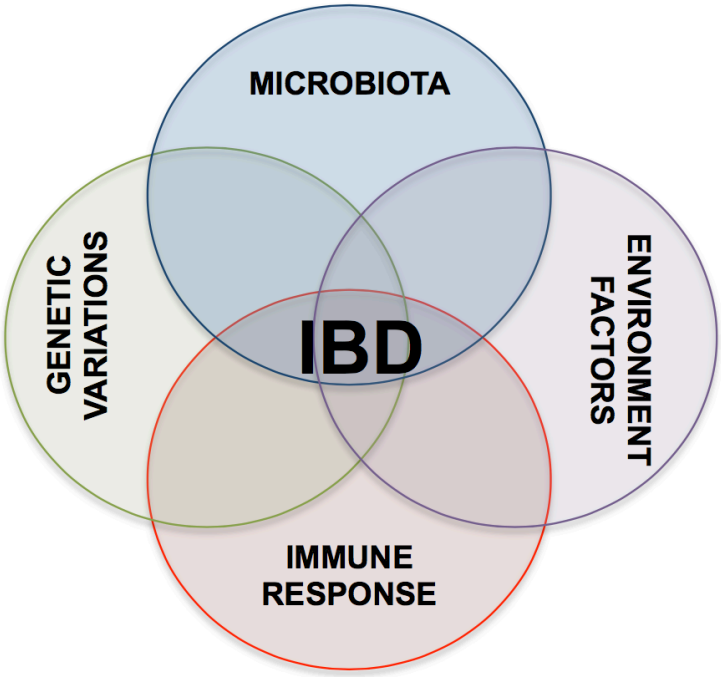


Figure 1. Factors involved in Inflammatory Bowel Disease (IBD)

1. Genetic variations

Since the original description of "terminal ileitis" by Crohn and collaborators in 1932, we have needed a long time to properly determine the occurrence of IBD, and establish the existence of a genetic basis (Kirsner & Spencer, 1963). This was uncovered after the first genome scan when it was found a link of CD with chromosome 16 (Hugot et al., 1996). Shortly after, the first IBD gene was discovered, the *intracellular nucleotide oligomeration domain 2/caspase recruitment domain 15* (*NOD2/CARD15)* gene variants associated with ileal CD (Hugot et al., 2001). Recently, a high number of genome-wide association studies (GWAS) have published many genes associated with IBD, but preliminary reports with increasingly fine sequencing power indicate that only a few additional ones remain to be discovered. Although the genetic component is stronger in CD than in UC, approximately 30% of these IBD-related genetic loci are shared between both conditions (of the 163 gene variants associated with IBD, 23 are associated with UC, 30 with CD, and the remaining 110 with both UC and CD), indicating that these diseases engage common pathways (Franke et al., 2010; Jostins et al., 2012).

It is now evident that gene variants are implicated in IBD pathogenesis. In fact, recent studies show that the odd ratio for developing UC or CD increases in direct proportion to the number of risk alleles that each patient carries (Wang et al., 2014). Analyses of the genes and genetic loci implicated in IBD show several pathways that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, regulation of adaptive immunity, endoplasmic-reticulum (ER) stress and metabolic pathways associated with cellular homeostasis.

Several genetic analyses have demonstrated that there are 7 loci (inflammatory bowel disease 1-7, IBD1-7) involved with the susceptibility to disease, and most of them have focused the research on mutations of genes like *NOD2/CARD15*, *MHC-II* (*major histocompatibility complex-II*), cytokines, cytokine receptors and adhesion molecules (Sartor, 2003; Zheng, Hu, Zeng, Lin, & Gu, 2003).

NOD2/CARD15, located on chromosome (Chr) 16q12 (IBD1), was the first specific gene to be associated with IBD, in particular with ileal CD in white (but not oriental) populations (Hugot et al., 2001; Ogura et al., 2001). This gene is expressed on immune cells (monocytes, macrophages and dendritic cells), although there are evidences of its low expression in epithelial cells. It is strongly induced by different inflammatory stimuli, including some bacterial components (Berrebi et al., 2003; Rosenstiel et al., 2003). Muramyl dipeptide (MDP), found in bacterial peptidoglycan, is recognized by the leucine rich repeat (LRR) domain of NOD2 and leads to the activation of Nuclear factor-kappaB (NF-κB) through a receptor-interacting serine-threonine kinase-2 (RIPK2)-dependent signalling pathway (Inohara, Chamaillard, McDonald, & Nunez, 2005; Kobayashi et al., 2002). Moreover, mutations in *NOD2* result in decreased production of antibacterial defensins by Paneth cells (Kobayashi et al., 2005; Wehkamp, Harder, Weichenthal, et al., 2004), and therefore, patients with this mutation have defective clearance of intracellular bacteria in intestinal epithelia (Kamada et al., 2005) and also impaired immune responsiveness to bacterial components (Bonen & Cho, 2003). The inflammation developed in murine models exhibiting *CARD15* mutation has been also suggested to be driven by altered toll-like receptor (TLR) activation of NF-κB (Bonen & Cho, 2003). Low concentrations of MDP could impair generation of interleukin (IL)-8, tumour necrosis factor (TNF)-α and IL-1β, similarly to the deficient signalling through TLRs when the CD-associated variants are present. This diminished early innate immune response could lead to inadequate microbial clearance and eventually result in the characteristic inflammation of CD (van Heel et al., 2005). In fact, homozygous people for a *NOD2* variant may have a ≥ 20-

fold increase in susceptibility to CD, although heterozygotes are also at increased risk. However, it is important to note that only ≈20% of CD patients are homozygous for *NOD2* variants (Cuthbert et al., 2002). Later on, the *organic cation transporter 1* (*OCTN1*) *on* Chr *5* was identified as another susceptibility gene (Lamhonwah, Skaug, Scherer, & Tein, 2003), whereas other evidences have suggested that the *disks large homolog 5* gene (*DLG5*) on Chr 10, could be a third gene (Stoll et al., 2004). In addition, polymorphisms of the *TLR4* gene have been reported in both CD and UC (Franchimont et al., 2004), further reinforcing the notion of potentially defective innate immunity pathways in these patients, related with the recognition and response to bacteria.

Moreover, variants of the *IL-23 receptor* (*IL23R*) gene were found in both CD and UC (Duerr et al., 2006), being in this case protective (Momozawa et al., 2011). IL-10R signalling components have also been implicated, including *interleukin 10 receptor alpha* (*IL10RA)* polymorphisms, signal transducer and activator of transcription (*STAT)3*, tyrosine kinase (*TYK)2*, janus kinase (*JAK)2* and *IL-10* itself (E.-O. Glocker et al., 2009). An unsuspected role for autophagy in IBD was recently described, implicating two component genes, *autophagy related 16-like 1* (*ATG16L1)* and immunity-related guanine tri-phosphatase family M (*IRGM*) (Cadwell et al., 2008; Deretic, 2009; Kaser et al., 2008). Similarly, genetic variants that perturb mechanisms that protect against ER stress can signal apoptosis and can affect intestinal homeostasis in IBD (Goodall et al., 2010).

In addition to coding variants, non-coding single nucleotide polymorphisms (SNPs) have shown to confer susceptibility to CD, like the SNPs in tumour necrosis factor ligand superfamily (*TNFSF15)* (Thiebaut et al., 2009). Furthermore, genetic changes may affect transcription-factor-binding sequences, locus accessibility, translational efficiency and trans-regulators such as non-coding ribonucleic acids (RNAs) and micro-RNAs (miRNAs). In this regard, IBD-implicated loci contain more than 10 miRNA-encoding sequences and 39 large intervening non-coding RNAs (lincRNAs), supporting the notion that regulation of gene expression by miRNAs and lincRNAs may be mechanistically relevant in IBD (Khalil et al., 2009).

2. Environmental influences

Epidemiological studies have already identified the increasing global incidence and prevalence of IBD in developing countries as these countries become more

developed and “Westernised”, highlighting the significance of environmental factors on influencing IBD development globally (Yang, Loftus, & Sandborn, 2001). These studies have considered a large number of risk factors for developing IBD, such us cigarette smoking, diet, oral contraceptives, vaccination history and other drugs, appendectomy, infections, water supply, social circumstances and perinatal and childhood factors. However, even with the notable amount of studies already conducted on this matter, many of the suggested environmental risk factors for CD and UC still remain contentious when considering their exact relationship with these intestinal conditions (Molodecky & Kaplan, 2010).

One of the features that has been used to explain the increasing evidence of IBD is the “hygiene hypothesis”, which proposes that the lack of proper exposure to common infections early in life negatively affects the development of the immune system, which becomes less “educated” and less prepared to deal with multiple new challenges later in life (Strachan, 1989). This is indirectly supported by evidence of improved health and acquisition of western societies habits in parts of the world where IBD is emerging (Okada, Kuhn, Feillet, & Bach, 2010; Yazdanbakhsh, Kremsner, & van Ree, 2002).

3. Altered immune response

It is well known the important role of the well-evolved mucosal innate immune system, complemented by the intestinal epithelium that acts as a physical barrier, defending against pathogenic incursions, and limiting inflammatory responses to maintain a state of hypo-responsiveness to commensal bacteria. However, different studies have proposed that this innate immune system is also the effector arm in mediating intestinal inflammation. In fact, GWAS suggests that dysregulation in innate and adaptive immunity contributes to the development of IBD. The main issue is to know the specific antigenic determinant that triggers an abnormal immune response. Closely related with this concern, it has been reported in CD patients that a diversion of faeces induces inflammatory remission and mucosal healing in the downstream intestinal segment, whereas the infusion of faeces reactivates the disease (D'Haens et al., 1998). Furthermore, in UC patients with active disease, treatment with broad-spectrum antibiotics reduced mucosal inflammation (Casellas et al., 1998). All these data would support the concept that luminal bacteria could provide the stimulus for the development of the inflammatory response that leads to mucosal injury in human IBD. In consequence, the altered immune response that takes place in IBD may be caused,

directly or indirectly, by host microbiome, which, in a situation of a loss/weakness of barrier function, the normal immune regulation can be overwhelmed as a result of a dysfunction in the regulatory mediators of the intestinal immune system (Baumgart & Carding, 2007).

In fact, various components of the mucosal immune system have been implicated in the pathogenesis of IBD, including intestinal epithelial cells, macrophages/monocytes, neutrophils, dendritic cells (DCs) (innate immune system), T-cells and B-cells (adaptive immune system), as well as their secreted mediators (cytokines and chemokines). It has been proposed that an initial defect in sampling gut luminal antigens, or a mucosal susceptibility, leads to the activation of the innate immune response, most probably associated to an enhanced TLR activity. Then, antigen-presenting cells can mediate the differentiation of naïve T-cells into effector T helper (Th) cells, including Th1, Th2, and Th17 cell types, which in immune tolerance to commensal bacteria in the intestine (Duchmann et al., 1995; Macpherson, Khoo, Forgacs, Philpott-Howard, & Bjarnason, 1996).

3.a. Innate immunity

The innate immune system is the first line of defence, thus providing an immediate protective response against infections, and also helps to initiate the adaptive immune response. The innate immune system is non-specific and does not confer immunity memory, being comprised by the epithelial cell barrier, macrophages, monocytes, neutrophils, DCs, natural killer (NK) cells, eosinophils and basophils. These cells act together to initiate inflammation by secreting cytokines, chemokines and antimicrobial agents. Also, the surface of the intestine is protected by a layer of mucus that is generated by goblet cells in the epithelium. The inner mucus layer is approximately 100 μm thick, firmly adherent, rich in antimicrobials and mucin, and has a low bacterial density. The outer layer of mucus is comprised of mucin, diluted antimicrobials, and some bacteria. A variant in the *MUC2* gene, which is the major intestinal secretory mucin, confers susceptibility in humans to IBD and *MUC2* deficient mice develop spontaneous colitis (Van der Sluis et al., 2006). Furthermore, some patients with CD have been found to have goblet cell depletion and an impaired mucus layer, which allows bacteria to adhere directly to epithelial cells, and may contribute to disease progression (Larsson et al., 2011).

Traditionally, CD and UC have been viewed as predominantly T-cell-driven processes; however, more recent evidences suggest that innate immune responses could also play an important role, at least in CD, in initiating the inflammatory cascade and the subsequent pathological adaptive immune responses (Podolsky, 2002). Supporting this, patients with innate immunodeficiency tend to develop IBD and, similarly, patients with CD have defective innate immune responses, including attenuated macrophage activity in vitro, as well as impaired neutrophil recruitment and exogenous *Escherichia coli* clearance in vivo (Smith et al., 2009).

Although the intestinal network of DCs and macrophages are involved in local innate immune phenomena, these cells also have an important role in shaping adaptive immunity in response to intestinal environmental antigens (Coombes & Powrie, 2008; Rescigno, Lopatin, & Chieppa, 2008). Under homeostatic conditions, both DCs and macrophage populations have specific adaptations that promote tolerance, being conditioned by the mucosal microenvironment to express a non-inflammatory phenotype. Intestinal resident macrophages are highly phagocytic cells that clear apoptotic cells and debris and contribute to epithelium wound repair (Smith, Ochsenbauer-Jambor, & Smythies, 2005; Smith et al., 2011). However, they try to prevent excessive inflammatory responses towards the intestinal flora, including expression of inhibitors of NF-κB signalling that permit bactericidal activity in the absence of TLR-driven proinflammatory cytokine production (Smith et al., 2011), of by increasing IL-10 production and maintenance of forkhead box P3 (Foxp3) among colonic regulatory T (Treg) cells (Murai et al., 2009). Furthermore, intestinal DCs are highly specialized antigen presenting cells (APCs) that can provide protection and defence, induce tolerance or mediate inflammation (Bilsborough & Viney, 2004). For example, Treg-cell differentiation can be promoted by tolerogenic DCs (Rescigno & Iliev, 2009), whereas DCs expressing E-cadherin are a pro-inflammatory subset that promotes Th17-cell differentiation (Siddiqui, Laffont, & Powrie, 2010). Moreover, bacterial flagellins can stimulate TLR5 in hyporesponsive DCs from lamina propria and induce the release of pro-inflammatory mediators (Uematsu et al., 2006). Also, CD11chigh CD103+ DCs are dispersed throughout the lamina propria, taking up pathogenic and commensal bacteria, innocuous antigens or apoptotic intestinal epithelial cells (IECs); after maturation, they migrate to the draining mesenteric lymph node, where they initiate adaptive responses focused on the intestine, preferentially inducing Foxp3+ Treg cells (Coombes & Powrie, 2008; Varol et al., 2009). However, during intestinal inflammation, they acquire inflammatory properties, such as the ability to produce IL-6 and drive Th1 responses (Laffont, Siddiqui, & Powrie, 2010). Finally,

CD103– CX3CR1+ APCs comprise a heterogeneous population of DCs and macrophages, whereas CD11c+CX3CR1+ DCs are adjacent to the intestinal epithelium where they sample antigens and bacteria (Coombes & Powrie, 2008; Varol et al., 2009).

It has been reported that in both IBD and experimental colitis, there is an increase in DCs and macrophage populations displaying an activated phenotype, with enhanced expression of microbial receptors, that contributes to intestinal pathology through the potent pro-inflammatory effects of the cytokines that they secrete, particularly TNF-α and IL-6 (Hart et al., 2005; Varol, Zigmond, & Jung, 2010). In CD, they produce more IL-23 and TNFα than those in normal and UC mucosa, and contribute to the production of interferon (IFN)-γ by local T cells (Kamada et al., 2008). Acute and chronic mouse colitis models were also associated with a marked increase in recruited monocyte-derived DCs and macrophages that produced IL-12, IL-23 and TNF-α and showed enhanced TLR responsiveness (Platt, et al., 2010; Varol et al., 2009).

Other cells clearly involved in the inflammatory process that occurs in IBD are neutrophils. In fact, neutrophil accumulation in the intestinal mucosa, mainly within epithelial crypts, directly correlates with clinical disease activity and epithelial injury in human IBD. Thus, activated neutrophils, through their myeloperoxidase activity, produce reactive oxygen and nitrogen species, which induce oxidative stress within intestinal mucosa that participates in the tissue damage associated to these conditions (Chin & Parkos, 2006). However, neutrophils also contribute to the resolution of inflammation, by synthesizing anti-inflammatory mediators such as lipoxin A4; studies showing impaired secretion of lipoxin A4 in mucosal tissues from UC patients support the relevance of such mechanisms in IBD (Narushima et al., 2008).

In addition, innate leukocyte populations, including γδ T cells, natural killer T (NKT) cells and NK cells, can secrete Th1- and Th17- dervied cytokines such as IFN-γ, IL-17a and IL-22 (Colonna, 2009; Cua & Tato, 2010; Maloy & Kullberg, 2008; Wolk, Witte, Warszawska, & Sabat, 2010), thus also contributing to intestinal inflammation.

3.b. Adaptive immunity

- T cells.

Mucosal CD4+ lymphocytes play a central role in both the induction and the maintenance of the activated immune response that characterizes human IBD. Initially,

different studies revealed that cytokines associated with Th1 cell activity, including TNF-α, IFN-γ or IL-12 were markedly increased in inflamed mucosa from CD patients; whereas the cytokine profile in inflamed areas of UC seemed to exhibit increased production of the Th2 cytokines, like IL-5, IL-13 and transforming growth factor (TGF)-β. Moreover, it is well reported that these cytokines are potent in vitro stimulators of intestinal mucosal effector functions, including T cell and macrophage proliferation, adhesion molecule and chemokine expression, as well as the secretion of other pro-inflammatory cytokines, thus generating a vicious circle that collaborates to maintain the inflammatory response (Sartor, 2006; Shih & Targan, 2009).

However, after the identification and characterization of a new Th subset in the intestinal mucosa from CD patients, the IL-17-producing Th17 cells as well as the IL-23-mediated expansion of IL-17-producing T cells (Annunziato, et al., 2009; Annunziato et al., 2007), recent studies have suggested that the alterations initially attributed to Th1 and Th2 populations could result from the down-regulating effects of their products on this new Th cell population. Furthermore, the microbiota has been proposed to play an important role in the preferential localization of Th17 cells in the gut (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Th17 cells require specific cytokines and transcription factors for their differentiation, and although the function of this cell type has not been completely elucidated, emerging data suggest that these cells may play an important role in host defence against extracellular pathogens, which are not efficiently cleared by Th1 or and Th2-type immunity. Regarding the relative enrichment of Th17 cells at mucosal sites, together with the increased levels of Th17 cytokines in the inflamed gut (Ahern et al., 2010; Maloy & Kullberg, 2008), tissue destruction might therefore actually be mediated by these Th17 cells subset (Stockinger, Veldhoen, & Martin, 2007; Wynn, 2005). Furthermore, these cells are also involved in the proliferation, maturation and chemotaxis of neutrophils, thus contributing to the pathogenesis of these intestinal conditions (Matsuzaki & Umemura, 2007).

In addition to Th cell activation, it has been proposed that reduced numbers of Treg cells, characterized by CD4+, CD25+ and Foxp3+, which produce IL-10 and/or TGFβ, might be equally important in the pathogenesis of IBD, since these cells monitor the immune response and prevent an excessive and potentially harmful immune activation (Feuerer, Hill, Mathis, & Benoist, 2009; Jiang & Chess, 2004). Although there are various T-cell populations, Foxp3+ Treg cells and Foxp3− IL-10- secreting CD4+ T cells are particularly important in the intestine (Izcue, Coombes, & Powrie, 2009). Although they are usually generated in the thymus, the small intestine and

colon are also a preferential sites for TGF-β-dependent induction of Foxp3+ Treg cells, where they control potentially deleterious responses to dietary and microbial stimuli (Feuerer et al., 2009). In fact, microbiota has also got a role in promoting intestinal Treg-cell responses, since Treg-cell accumulation in the colon is reduced in germ-free mice and can be increased by incorporation of particular indigenous bacteria (Atarashi & Honda, 2011). Supporting the important role of these cells, it has been described that the deletion or loss-of-function mutations in the gene encoding Foxp3 result in inflammatory disease in mice and humans, often accompanied by intestinal inflammation (Izcue et al., 2009).

Interestingly, induced Treg and Th17-cell populations seem to be reciprocally regulated in the intestine. Although TGF-β is required for the differentiation of both populations, the presence of STAT3-mediated signals (such as IL-6 or IL-23) promotes Th17 cells at the expense of Foxp3+ Treg cells (Ahern et al., 2010; Littman & Rudensky, 2010). This mechanism allows the inflammatory response to override Treg-cell induction in the presence of pro-inflammatory stimuli, promoting intestinal effector T-cell responses and host defence. In fact, mice with a *Stat3* deletion in Foxp3+ Treg cells develop aggressive colitis due to uncontrolled Th17 response (Chaudhry et al., 2009). This system is precisely balanced but sometimes it can lead to Treg-cell deregulation. For example, high-level T-bet expression in the presence of acute intestinal infection drives Treg cells into an inflammatory IFNγ-secreting phenotype (Oldenhove et al., 2009). Transcription factors that direct Th1-cell or Th17-cell responses, such as T-bet or retinoic-acid- receptor-related orphan receptor-γt (RORγt), respectively, were shown to be essential for T-cell-mediated colitis (Leppkes et al., 2009).

- B cells.

Antibody (Immunoglobulin(Ig)M, IgG and IgA) synthesis and secretion have been reported to be altered in active IBD, both in the circulation and at the mucosal levels (MacDermott et al., 1981). However, the patterns of antibody production differ in UC and CD, particularly in regard to IgG production: in UC there is a disproportional increase in IgG1 secretion, while in CD IgG1, IgG2 and IgG3 are increased (Scott et al., 1986). Although a limited attention has been paid to B cells in IBD, a renewed interest could occur if new biologicals that specifically induce B cell depletion, like rituximab, turn out to be effective in the management of these intestinal pathologies (Perosa, Prete, Racanelli, & Dammacco, 2010).

3.c. Inflammatory mediators

IBD, similarly to other inflammatory conditions, is characterized by the involvement of a broad spectrum of inflammatory mediators, including cytokines, chemokines, leukotrienes and prostaglandins, which actively participate in all the phases of the inflammatory process: initiation, progression and resolution, if it occurs.

Chemokines mediate the recruitment of leucocyte effector populations to the sites of immune reaction and tissue injury (Laing & Secombes, 2004). In chronic inflammatory diseases like IBD, aberrant leukocyte chemoattraction occurs, characterised by an excessive recruitment of inflammatory cells into the injured intestine (Fiocchi, 1998). Chemokines tightly control leukocyte adhesion and migration across the endothelium (Baggiolini, 1998), but they are also able to trigger multiple inflammatory actions including leukocyte activation, granule exocytosis, production of metalloproteinases for matrix degradation, and up-regulation of the oxidative burst (MacDermott, Sanderson, & Reinecker, 1998; Papadakis & Targan, 2000). During the active phases of IBD, some chemokines are consistently increased: IL-8 and its receptor, monocyte chemoatractant protein (MCP)-1 and MCP-3, and macrophage inflammatory proteins (MIP) (Keshavarzian et al., 1999; Ohtsuka, Lee, Stamm, & Sanderson, 2001; Reinecker et al., 1995; Uguccioni et al., 1999). Similarly, the up-regulated expression in IBD of different adhesion molecules, such as the intercellular adhesion molecule (ICAM)-1, the lymphocyte function-associated antigen (LFA) -1, the macrophage 1 antigen (Mac-1), the vascular cell adhesion molecule (VCAM)-1, the very late antigen (VLA)-4 and P- and E-selectins, promotes the recruitment of granulocytes and lymphocytes through blood vessels. In addition, these adhesion molecules also facilitate cell interactions, like those between lymphocytes and APCs or among lymphocytes, thereby sustaining the immune-inflammatory response (Nakamura, Kobayashi, & Kato, 1993). Of note, ICAM-1 is pivotal for the influx of neutrophil granulocytes into colonic mucosa, and gene analyses have found polymorphisms in the gene encoding ICAM-1, indicating that it may be involved in the pathogenesis of UC (Vainer, 2010).

The roles of cytokines in IBD are very diverse and complex. The fact that these mediators control T-cell differentiation and regulation has made them to be considered as central points of potential intervention to control the inflammatory response. IL-12, IL-18 and IL-23 have a crucial function in Th1 differentiation and chronic activation, whereas other cytokines, such as TNF-α, IL-1β and IL-6 augment the inflammatory

response by recruiting other cells and enhancing the production of inflammatory mediators (Sanchez-Muñoz, Dominguez-Lopez, & Yamamoto-Furusho, 2008).

The T cell growth factor, or IL-2, is the major cytokine that is produced during the primary response of Th cells. This cytokine acts through its receptor, IL-2R, to activate signalling molecules that are involved in cell proliferation (Schimpl et al., 2002). In fact, IL-2 knockout (KO) mice have been reported to develop intestinal inflammation, which is dependent on both T-cell and the presence of intestinal microbiota (Sadlack et al., 1993); these mice were shown to be deficient in CD4+CD25+ Treg cells, thus promoting the expansion of organ-specific T cells, one of the major causes for UC (Claesson et al., 1999; Papiernik, de Moraes, Pontoux, Vasseur & Penit, 1998).

IL-23 is induced by pattern recognition receptors (PRRs), whose sustained activation drives chronic intestinal inflammation. ER stress can also synergize with TLR signals to selectively increase IL-23 expression by DCs (Goodall et al., 2010), which is constitutively expressed in a small population of DCs present in the lamina propria (Kamada et al., 2008). It was initially linked to the preferential expression of Th17 responses, but it can promote a wide range of pathological responses in the intestine, mediated either by T cells or by excessive innate immune activation. IL-23-mediated enhancement of Th1 and Th17 responses is consistent with the increased levels of IFN-γ, IL-17 and IL-22 observed in the chronically inflamed intestine (Ahern et al., 2010; Maloy & Kullberg, 2008). Furthermore, studies in several mouse IBD models have used selective targeting of the IL-23 p19 subunit to demonstrate that IL-23 plays a key part in chronic intestinal pathology (Maloy & Kullberg, 2008). T-cell-intrinsic IL-23R signals favour the expression of pathogenic pro-inflammatory T-cell responses in several ways, including enhanced proliferation of effector T cells, reduced differentiation of Foxp3+ Treg cells and the emergence of IL-17+IFN-γ+CD4+ T cells (Ahern et al., 2010), as found in the inflamed lamina propria of patients with CD (Cosmi et al., 2008).

The IL-17 cytokine family is a group of cytokines that includes at least six members: IL- 17A, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25) and IL- 17F; they act both in vitro and in vivo as potent pro-inflammatory cytokines (Kolls & Linden, 2004). IL-17 can induce the expression of pro-inflammatory cytokines (like IL-6 and TNF-α), chemokines (including keratinocyte chemoattractant (KC), MCP-1 and MIP-2) and matrix metalloproteases, which mediate tissue infiltration and tissue destruction (Park et al., 2005). Of note, the role of these cytokines has not been fully elucidated yet, since several studies have reported controversial results; for example, in acute dextran

sodium sulphate (DSS) colitis, IL-17A has a protective role, whereas IL-17F seems to exacerbate disease (Yang et al., 2008). However, in experimental models of T-cell-transfer colitis, IL-17A and IL-17F can have redundant pro-inflammatory effects in the gut (Leppkes et al., 2009). Moreover, in mice with *Stat3*-deficient Treg cells, the neutralization of IL-17A attenuated chronic colitis (Chaudhry et al., 2009) and decreased innate immune colitis after *Helicobacter hepaticus* infection (Buonocore et al., 2010). The IL-17 family also expresses IL-22, IL-21 and chemokine (C-C motif) ligand 20 (CCL20) (ligand of CC-chemokine receptor 6). IL-22 is mainly produced by innate lymphoid cells (ILCs) expressing the transcription factor RORγt (Cella et al., 2009; Sawa et al., 2011), and the involvement of IL-22 in chronic intestinal inflammation has been supported by the findings describing that colonic and serum IL-22 levels are increased in IBD patients, as well as in several mouse models of colitis (Sugimoto et al., 2008; Wolk et al., 2007). Conversely, IL-22 is emerging as an important cytokine in epithelial homeostasis, showing protective activity in different models of colitis through its stimulatory effect on antimicrobial and reparative processes. Thus, in IECs, IL-22 signalling drives the production of antimicrobial peptides (AMPs) and promotes epithelial regeneration and healing by activating the transcription factor STAT3 (Pickert et al., 2009). Consistent with this, IL-22 administration attenuated disease severity in the DSS and T-cell receptor-α (Tcra−/−) mouse experimental models, by restoring goblet cells and mucus production (Wolk et al., 2010).

Although less extensively studied, IL-21 is mainly produced by activated CD4+ T cells, specially the Th17 subset (Korn, Oukka, Kuchroo, & Bettelli, 2007; Nurieva et al., 2007; Parrish-Novak et al., 2000; Zhou et al., 2008), but NKT cells (Coquet et al., 2007; Harada et al., 2004) and T follicular helper cells (Bryant et al., 2007; Chtanova et al., 2004) can also secrete this cytokine. IL-21 may regulate intestinal inflammation through effects on Th17 cells and the production of matrix metalloproteinases (MMPs) (Maloy & Kullberg, 2008). IL-21-stimulation in colonic epithelial cells has resulted in enhanced synthesis of the T-cell chemoattractant MIP-3α/CCL20 (Caruso et al., 2007) and in vivo blockade of this chemokine attenuated lymphocyte recruitment into the intestine in DSS-induced colitis (Teramoto et al., 2005). Another mechanism by which IL-21 may increase or sustain pro-inflammatory responses in the intestinal tract is by enhancing IFN-γ production by T cells and NK cells (Strengell et al., 2003; Strengell et al., 2002). Indeed, IL-21 has been reported to promote Th1 responses in biopsies from CD (Monteleone et al., 2005); however, under other circumstances, IL-21 has been reported to have an inhibitory effect on IFN-γ expression and to promote Th2

responses (Pesce et al., 2006; Suto, Wurster, Reiner, & Grusby, 2006; Wurster et al., 2002), suggesting that its effects on T-cell responses may be context-dependent. Taken together, the pleiotropic effects of IL-21 may contribute to chronic intestinal inflammation, augment pathogenic leukocyte responses in the gut (mainly those mediated by Th1 and Th17 cells, and potentially also by NK cells) and may exacerbate inflammation through its effects on tissue cells in the gut, stimulating the production of T-cell chemoattractants and inducing the release of tissue-degrading matrix MMPs.

The association of IBD with polymorphisms in NOD-like receptor (NLR) family, NOD-like receptor pyrin domain (NLRP) 3 and IL-18 receptor accessory protein (IL18RAP), together with the central role for inflammasomes and NLRs in auto-inflammatory diseases (Schroder, Zhou, & Tschopp, 2010) have promoted the interest in defining the potential roles of IL-1β and IL-18 in IBD. It has been reported that their levels are increased in human IBD (Kaser, Martinez-Naves, & Blumberg, 2010; Siegmund, 2010), and IL-18$^{-/-}$ mice have been described to be resistant to the experimental colitis induced by trinitrobenzene sulphonic acid (TNBS) (Salcedo et al., 2010), thus suggesting their contribution to the intestinal pathology. This hypothesis is consistent with the ability of IL-1β and IL-18 to promote Th17 and Th1 responses, respectively (Siegmund, 2010). Supporting this, it has been shown that the suppression of NF-κB signalling and NLRP3 inflammasome activation resulted in the amelioration of experimental colitis in mice (Wu et al., 2014).

A wide range of leukocytes, including T cells, B cells and myeloid cells, expresses the cytokine IL-10. The colon contains large numbers of CD4+ IL-10+ cells, mainly Foxp3+, whose IL-10 production is required to prevent intestinal inflammation. In fact, IL-10-/- mice spontaneously develop colitis (Izcue et al., 2009). Besides, intestinal microbiota can promote the activity of colonic Treg cells by inducing IL-10 production (Atarashi & Honda, 2011). Foxp3−IL-10+ CD4+ cells are more heterogeneous since most effector Th-cell subsets produce IL-10 after chronic immune stimulation (Saraiva & O'Garra, 2010). Myeloid sources of IL-10 are also important in some settings, as shown in an adoptive transfer model of colitis in which IL-10 production by intestinal macrophages promoted Foxp3 Treg-cell function (Murai et al., 2009). Moreover, this cytokine controls chronic intestinal inflammation partly through direct anti-inflammatory effects on myeloid cells (Izcue et al., 2009). An evidence for the role of IL-10 in human IBD comes from the findings those mutations in the IL-10 receptor (IL10R) genes *IL10RA* and *IL10RB* lead to severe early-onset IBD (Glocker et al., 2009).

TGF-β is another cytokine produced by multiple cells types. It is an inhibitory cytokine recognized as a key regulator of immunological homeostasis and inflammatory responses in the gut (Li & Flavell, 2008). For example, it stimulates intestinal IgA responses, thus reinforcing intestinal homeostasis (Cong, Feng, Fujihashi, Schoeb, & Elson, 2009). During inflammation, TGF-β in the presence of pro-inflammatory cytokines such as IL-6 promotes the development of inflammatory Th17 responses (Torchinsky, Garaude, Martin, & Blander, 2009).

3.d.miRNAs

miRNAs are single stranded of noncoding RNAs, on an average of 22 nucleotides long, highly conserved throughout evolution and discovered in all eukaryotic cells except fungi (Lee et al., 2010; Niwa & Slack, 2007). miRNAs bind to complementary 3′ untranslated regions (UTRs) of targeted protein-encoding messenger RNAs (mRNAs), resulting in decreased stability and repression of translation. The interest for miRNA research is expanding rapidly, since the first discovery of miRNA in 1993 (Lee, Feinbaum, & Ambros, 1993; Wightman, Ha, & Ruvkun, 1993), there have been described over 2500 mature human miRNA transcripts (Kozomara & Griffiths-Jones, 2014). Recent investigations into the biological functionality of miRNAs have discovered their ability to adapt to physiologic and pathophysiologic environmental stress, as well as to restore or alter gene expression in different tissues, thus exerting epigenetic post-transcriptional effects on gene regulation (Leung & Sharp, 2010). Also, each miRNA can target hundreds of genes, and a particular gene is usually the target of multiple miRNAs, adding complexity to the regulation of the gene transcriptional network (Rebane & Akdis, 2013). In fact, it has been reported that miRNAs play an important role in many biological processes, such as a signal transduction, cellular proliferation, differentiation, apoptosis and immune response (Bushati & Cohen, 2007; O'Connell, Rao, Chaudhuri, & Baltimore, 2010). Furthermore, miRNAs have been recognized as critical components in the regulation of the innate and immune responses, and changes in miRNAs expression are related to many autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, psoriasis and IBD (Amarilyo & La Cava, 2012; Filkova, Jungel & Gay, 2012; Pekow & Kwon, 2012; Schneider, 2012), as well as others like neurological or cardiovascular diseases and cancer (Ikeda et al., 2007).

The key role played by miRNAs in IBD has been evidenced both in humans and in experimental models of colitis (Table 1). Regarding the later, loss of intestinal miRNAs in mouse models has been shown to impair differentiation of intestinal cells and epithelial barrier function, resulting in inflammation (Abraham & Cho, 2009). In 2008, Wu and colleagues completed the first miRNA profiling study to examine miRNA expression in colonic mucosal samples from IBD patients (Wu et al., 2008). They identified 11 miRNAs that were significantly modified in UC in comparison with control specimens, and demonstrated an inverse relationship between miR-92 and macrophage inflammatory peptide-2α, which it was previously shown to be implicated in IBD (Wu et al., 2008). Similarly, Bian et al. (2011) reported that miR-150 was differently expressed in inflamed colonic mucosa of UC patients, as compared to controls, establishing an inverse correlation between this miRNA and its target, a proto-oncogene, c-Myb (transcriptional activator myeloblastosis), that is involved in apoptosis (Xiao et al., 2008). Consequently, these studies have exposed new and important insights into the pathogenesis of IBD, and supported by additional studies focusing on deregulated miRNA expression and function (Takagi et al., 2010; Yang et al., 2013).

It has been previously commented that IL-23 acts on the IL-23R and promotes expansion and maintenance of Th17 cells, which have been implicated in the pathogenesis of IBD (Geremia & Jewell, 2012). Closely related to this, miRNAs have been considered as crucial mediators in regulating the IL-23/Th17 pathway, and the subsequent downstream IL-17 production in IBD. Xue et al. (2011) observed much lower expression of miR-10a in intestinal epithelial cells and dendritic cells of specific pathogen-free mice compared to germ-free mice. It has been identified IL12/IL-23p40 as a target of miR-10a. This suggested that microbiota negatively regulated host miR-10a expression by targeting IL12-IL-23p40, which could contribute to the maintenance of intestinal homeostasis.

It is known that the intestinal mucosal barrier maintains a delicate balance between the absorption of essential nutrients and the prevention of the entry, and the subsequent response, of harmful agents. In fact, it has been extensively reported that one of the initial steps that occurs in IBD is a dysfunction of the intestinal epithelial barrier. Different studies have shown the role of different miRNAs in IBD by impairing intestinal barrier function. Yang et al. (2013) found high expression of miR-21 both in the intestinal mucosa and serum of UC patients (Yang et al., 2013). The target of miR-21 is ras homolog family member B (RhoB), which is involved in modulating intestinal epithelial permeability and was found significantly decreased in UC patients. They

demonstrated that over-expression of miR-21 in patients with UC, and in Caco-2 cells as well, impaired intestinal tight junction integrity and morphology through targeting RhoB. Similarly, and confirming these observations, miR-21 has been reported to be over-expressed in IBD patients, IL-10 knockout (KO) mice and DSS-treated mice. In fact, miR-21 KO mice were less susceptible to experimental colitis and had a reduced inflammatory response than wild type mice (Shi et al., 2013).

In the intestinal epithelium, autophagy is considered as a defensive strategy for the clearance of intracellular microorganisms, and the impairment of autophagy results in intestinal epithelial dysfunction that contributes to IBD pathogenesis (Patel, 2013). Two genes associated with autophagy, *ATG16L1* and *IRGM* have been identified as CD susceptibility genes by GWAS (Hampe et al., 2007; Parkes et al., 2007). Different miRNAs, including miR-106b and miR-93, which target *ATG16L1,* can reduce autophagy in epithelial cells. In fact, the increased expression of miR-106b has been described to inhibit autophagy-dependent clearance of CD-associated adherent-invasive *Escherichia coli* (AIEC) in epithelial cells (Lu et al., 2014). Furthermore, inflamed mucosa from active CD patients showed over-expression of miR-106b and a lower expression of *ATG16L1* when compared with controls (Lu et al., 2014). These results reveal that the down-regulation of *ATG16L1* expression mediated by miR-106b and miR-93 in CD patients might manifest an altered antibacterial activity of associated intracellular bacteria in epithelial cells, and subsequently affected the outcome of intestinal inflammation. Moreover, it has been proved that other miRNAs, like miR-30c and miR-130a can directly and negatively regulate the expression of *ATG5* and *ATG16L1*, respectively, as shown in non-inflamed or inflamed ileal CD biopsy specimens. Similarly, the expressions of miR-30c and miR-130a have been also inversely correlated with those of *ATG5* or *ATG16L1* in the intestinal epithelial cell line T84 when infected with the AIEC. The inhibition of autophagic activity by miR-30c and miR-130a increased AIEC persistence within T84 cells and enhanced pro-inflammatory cytokine production. Furthermore, it has been demonstrated that the in vivo inhibition of miR30c and miR-130a suppressed AIEC-induced down-regulation of *ATG5* and *ATG16L1* expression and increased autophagic activity, leading to more efficient intracellular bacteria clearance and decreased inflammation (Nguyen et al., 2014).

Furthermore, it has been proposed that miR-124 could play a key role in IL6/STAT3 signalling pathway, which has been considered as crucial in IBD. In fact, the inhibition of IL-6/STAT3 cascades results in the suppression of acquired immune mediated colitis (Sugimoto et al., 2008). miR-124 expression was significantly

decreased in colon tissues with UC and mice with experimental colitis, and the levels of STAT3 and its regulated genes were simultaneously up-regulated (Koukos et al., 2013). Thus, reduced levels of miR-124 might increase the expression and activity of STAT3 by direct binding, which could promote inflammation.

The role of miRNAs in the etiopathogenesis of IBD has been also related to the NF-κB pathway. Different studies have shown that the transcription factor NF-κB is markedly induced in IBD patients, and strongly influences the course of mucosal inflammation through its ability to promote the expression of various pro-inflammatory genes (Atreya, Atreya, & Neurath, 2008). miR-146a has been reported to regulate gut inflammation via NOD2-sonic hedgehog (SHH) signalling, which is an important pathway in maintaining gut homeostasis and development. NOD-2 induced miR-146a target *NUMB*, a negative regulator of SHH signalling, thus alleviates the suppression of SHH signalling and subsequently increasing the pro-inflammatory cytokines expression (Ghorpade et al., 2013). Otherwise, the up-regulation of miR-126 may contribute to the pathogenesis of UC by targeting IκBα. Feng et al., (2012) found miR-126 was significantly increased tissue from patients with active UC compared to healthy controls, while in turn inhibitor protein κB alpha (IκBα) was down-regulated. Therefore, miR-126 could activate NF-κB signalling pathway by targeting IκBα and contribute to the development of UC (Feng et al., 2012). Furthermore, it has been proposed that the inhibition in the expression of cell adhesion molecules (CAMs), such as ICAM-1 and VCAM-1, could be also obtained after increased expression of miR-126 (Ghosh & Panaccione, 2010; Harris et al., 2008). Moreover, studies performed in lipopolysaccharide(LPS)-stimulated human colon derived CCD-18Co myofibroblast cells, in which the inhibition of NF-κB results in down-regulation of a wide range of downstream pro-inflammatory genes including TNF-α, IL-6 and CAMs, showed that up-regulating of miR-126 protects human colon cells from inflammation through targeting VCAM-1 (Angel-Morales, Noratto, & Mertens-Talcott, 2012). Likewise, altered expression of miR-122 has been reported to be associated with CD progression (Kanaan et al., 2012). Over-expression of miR-122 in HT-29 cells is associated with reduced apoptosis and down-regulated NOD2 expression when induced after incubation of these epithelial cells with LPS.

It has been also proposed that miR-122 might decrease intestinal epithelial cell injury in CD by targeting NOD2. The involvement of miR-122 in the regulation of intestinal epithelial tight junction (TJ) (Turner, 2009) has been confirmed in experiments conducted in TNF-α-stimulated Caco-2 cells, in which an increase in Caco-2 TJ permeability takes place by targeting occluding. The up-regulation of

intestinal permeability by miR-122 was proved in vivo as well and these two different studies showed a complex and controversial role of miR-122 in the development of IBD (Ye, Guo, Al-Sadi, & Ma, 2011).

Of note, NF-κB was originally thought to be an almost exclusively pro-inflammatory player in IBD setting, but its role in epithelial cells has been shown to be more controversial. Some studies using KO mice with defective NF-κB activation have demonstrated an anti-inflammatory function of NF-κB in colonic epithelial cells (Hayden, West, & Ghosh, 2006; Pasparakis, Luedde, & Schmidt-Supprian, 2006). A member of miR-146 family, miR-146b, can alleviate intestinal injury and increased the survival rate in DSS-induced mouse colitis via the activation of NF-κB and the subsequent improvement of epithelial barrier function (Nata et al., 2013). The beneficial effect is probably obtained after suppressing Siah2, the target of miR-146b, which promotes ubiquitination of TNF receptor associated factor (TRAF) proteins upstream of NF-κB.

Table 1. List of altered miRNAs involved in inflammatory bowel disease and their mRNA targets

miRNA	Target mRNA	Effect	References
Decreased expression			
miR-10a	IL12/IL23p40	Regulates intestinal homeostasis	Xue et al., 2011
miR-124	STAT3	Promotes inflammation	Koukos et al., 2013
miR-200b	SMAD2	Regulates epithelial-mesenchymal transition	Chen et al., 2013
miR-192, miR-495	NOD2	NF-κB signalling upregulation	Chuang et al., 2014
Increased expression			
miR-146a	NUMB	SHH signalling upregulation	Ghorpade et al., 2013
miR-146b	Siah2	NF-κB signalling upregulation	Nata et al., 2013
miR-126	IκBα	NF-κB signalling upregulation	Feng et al., 2012
	VCAM-1	Suppresses pro-inflammatory cytokines	Angel-Morales et al., 2012
miR-122	NOD2	Decreases intestinal epithelial cell injury	Chen et al., 2013
	Occluding	Intestinal permeability upregulation	Ye et al., 2011
miR-21	RhoB	Impairment of tight junction	Yang et al., 2013; Shi et al., 2013
miR-130a	ATG16L1	Inhibition of autophagic activity	Nguyen et al., 2014
miR-150	C-Myb	Promotes apoptosis	Bian et al., 2011
miR-106b	ATG16L1	Deregulation of autophagy	Lu et al., 2014; Zhai et al., 2013

MICROBIOTA

The term microbiota refers to the group of microorganisms that colonizes a given location of the human body, establishing a commensal relationship with the host. Actually, the gut microbiota constitutes the most complex ecosystem, and includes a few eukaryotic fungi, viruses, and some archaea, being bacteriathe most prominent component (Power et al., 2014). Host–microbe interactions are bi-directional and occur primarily along surface of the intestinal mucosa.

The number of bacteria found throughout the gastrointestinal tract differs from the oesophagus to the rectum. Acid, bile and pancreatic secretions obstruct the colonization of the stomach and proximal small intestine by most bacteria, being the number of them low in these locations. However, there is a steady increase of bacterial concentration towards the distal segments of the intestine. In the ileum and in the terminal ileum, there are 10^8 to 10^9 bacteria per gram of content, whereas in the colon, there are 10^{12} to 10^{14} bacteria per gram (Biedermann & Rogler, 2015) (Figure 2). Therefore, the number of bacteria within the gut is about 10 times that of all cells in the human body and includes ≈500-1,000 species, whose collective genomes, called microbioma, are estimated to contain 100 times more genes than our own human genome (Savage, 1977; Xu & Gordon, 2003). In addition to variations in the composition of the microbiota along the axis of the gastrointestinal tract, surface-adherent and luminal microbial populations also differ (Eckburg et al., 2005), and the ratio of anaerobes to aerobes is lower at the mucosal surfaces than in the lumen.

At birth, the entire gastrointestinal tract is sterile; the bacterial colonization is initiated during labour, when the first microbial exposure occurs. Different factors are known to influence colonization, including gestational age, mode of delivery (assisted versus vaginal delivery), diet (breast milk versus formula), level of sanitation and exposure to antibiotics (Fouhy et al., 2012; Marques et al., 2010). Initially, the colonization is characterized by the predominance of facultative anaerobes, enterobacteria and enterococci. These bacteria consume the oxygen and are gradually associated with anaerobic bacteria such as *Bifidobacterium, Bacteroides, Eubacterium, Veillonella* or *Clostridium.* The intestinal microbiota of newborns is characterized by low diversity and a relative dominance of the phyla *Proteobacteria* and *Actinobacteria*; thereafter, the microbiota becomes more and more diverse with appearance of the supremacy of *Firmicutes* and *Bacteriodetes*, which characterizes the adult microbiota (Backhed et al., 2004; Eckburg et al., 2005; Qin et al., 2010). It has been reported that these pioneering bacteria can modulate gene expression in the

host to create a suitable environment for themselves, thus preventing the growth of other bacteria that may be introduced later to the ecosystem (Xu & Gordon, 2003). By the end of the first year of life, the microbial profile is distinct for each infant, and it is considered that the composition and metabolism of the child intestinal microbiota maturates after two years, and becomes quite similar to that shown in the adult period of life (Guerin-Danan et al., 1997). There are many evidences that propose that disruption of the microbiota during this period of the maturation may be critical for disease occurrence in later life (Claesson et al., 1999; Clemente, Ursell, Parfrey, & Knight, 2012; de Meer, Janssen, & Brunekreef, 2005; Duramad et al., 2006; Gori et al., 2008; Han et al., 2009; Penders, Stobberingh, van den Brandt, & Thijs, 2007).

Anaerobic genera	Aerobic genera
Bifidobacterium	***Escherichia***
Clostridium	***Enterococcus***
Bacteriodetes	***Streptococcus***
Eubacterium	***Klebsiella***

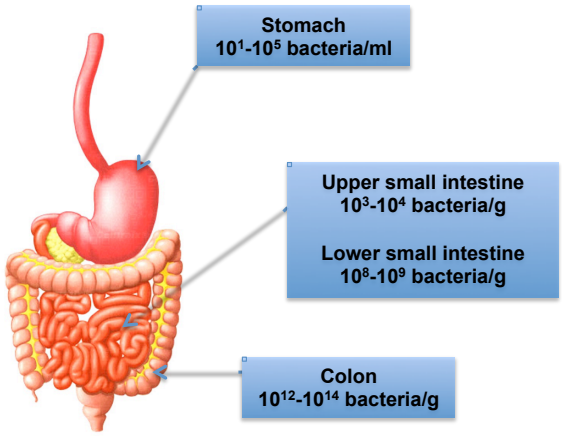


Figure 2. Numbers of bacteria per segment of the gastrointestinal tract in healthy individuals. Bacteria abundance increases in the jejunum/ileum from the stomach and duodenum, and in the large intestine. The most common anaerobic and aerobic genera are listed

Following infancy, the gut microbiota composition remains relatively constant, and although highly variable among different subjects, it has been observed that the composition of each individual's microbiota is so distinctive that it could be used as an alternative to fingerprinting (Claesson & O'Toole, 2010; Palmer et al., 2007). In fact, and more recently, 3 different enterotypes have been described in the adult human microbiome. *Prevotella*, *Ruminococcus,* and *Bacteroides* dominate these distinct enterotypes and their appearance seems to be independent of sex, age, nationality, and body mass index (Arumugam et al., 2011).

However, during human life, the microbiota composition is influenced not only by age but also by diet and socioeconomic conditions. In a recent study performed in elderly population, the interaction of diet, age and health status has been

demonstrated to play a key role (Claesson et al., 2012). When considering the diet, it is evident the contribution of nondigestible components of the diet to bacterial metabolism; however, the impact of other diet components on microbiota composition are now being explored. For example, data indicating a potential role of certain products of bacterial metabolism in colon carcinogenesis have already provided strong suggestions of the relevance of diet-microbiota interactions to disease (Claesson et al., 2012).

The importance of a particular bacterial composition relies on the fact that gut-commensal microbiota forms a natural defence barrier and exerts numerous protective, structural and metabolic effects on the epithelium (Figure 3). Their interactions play a fundamental role in promoting homeostatic functions such as immunomodulation, cytoprotection, regulation of apoptosis, as well as maintenance of barrier function (Patel & Lin, 2010). The important role attributed to gut microbiota on the development of gut function has been confirmed when germ-free animals are studied: these are more susceptible to infections, showing reduced vascularity, digestive enzyme activity, muscle wall thickness, cytokine production and serum immunoglobulin levels; in addition, Peyer's patches are smaller and there are fewer intraepithelial lymphocytes, but an increased enterochromaffin cell area is observed (Shanahan, 2002).

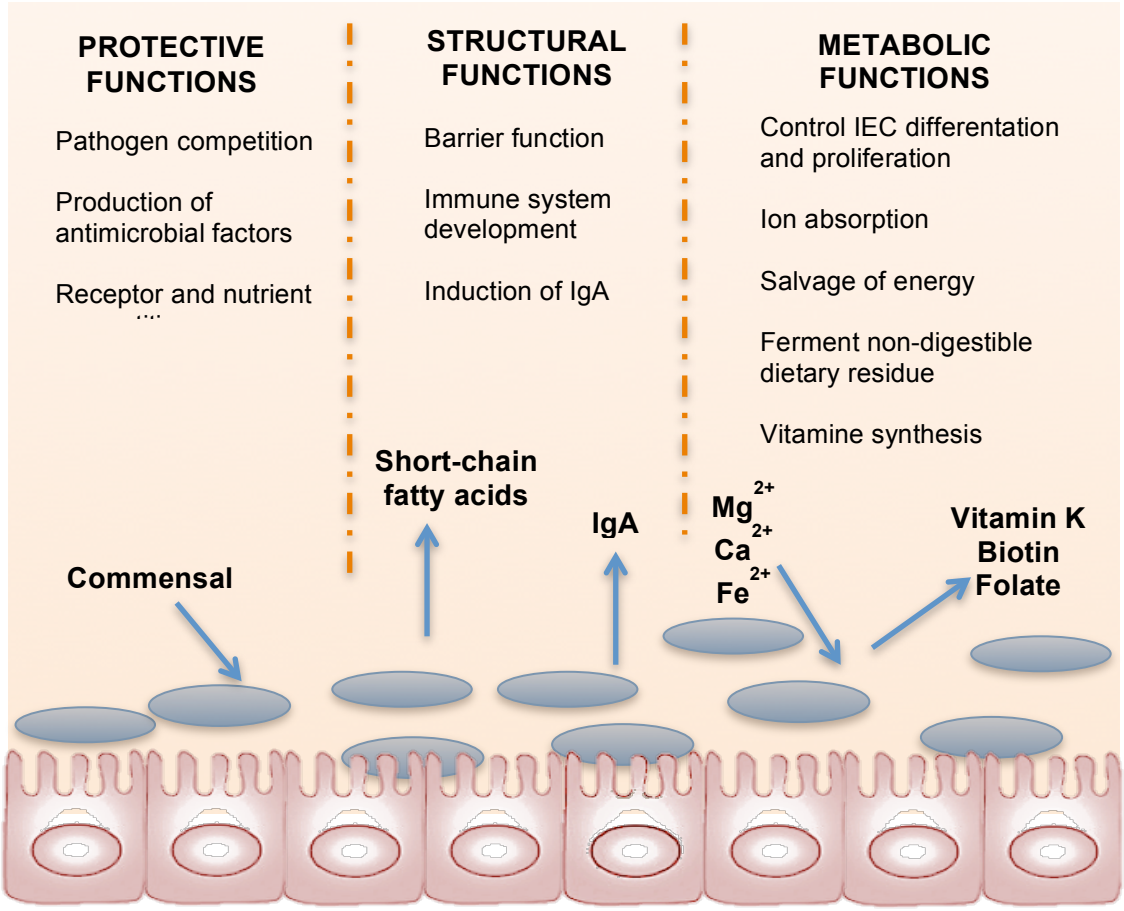


Figure 3. Commensal bacteria exert a miscellany of protective, structural and metabolic effects on the intestinal mucosa.

It is important to note that host defence requires an accurate interpretation of the microenvironment to distinguish commensal organisms from episodic pathogens, as well as a precise regulation of subsequent responses. The epithelium provides the first line of defence and tolerates commensal organisms through numerous mechanisms. These include the masking or modification of microbial-associated molecular patterns that are usually recognized by PRRs, such as TLRs (Lebeer, Vanderleyden, & De Keersmaecker, 2010) and NOD/CARD (Cario, 2005), and the inhibition of the NF-κB inflammatory pathway (Neish et al., 2000). It has been well reported that PRRs have a key role in immune-cell activation in response to specific microbial-associated molecular patterns. For example, TLR2 is activated by peptidoglycan and lipotechoic acids, TLR4 by lipopolysaccharide, TLR5 by flagellin; whereas NOD1/CARD4 and NOD2/CARD15 function as intracellular receptors of peptidoglycan subunits (Cario, 2005). Decreased enterocyte proliferation and levels of cytoprotective factors have been observed in TLR-defective mice, and TLR signals mediated by commensal bacteria or their ligands are essential for intestinal barrier function and repair of the gut (Fukata et al., 2005; Rakoff-Nahoum et al., 2004).

Different studies suggest that NOD2 can modulate signals transmitted through TLR3, TLR4 and TLR9 (Netea et al., 2005; van Heel et al., 2005). Although wild-type NOD2 activates pro-inflammatory signals, stimulation of NOD2 with peptidoglycan has been shown to inhibit TLR2-driven Th1 cytokine responses, and in the absence of NOD2, peptidoglycan triggers imbalanced TLR2-mediated cytokine production (Watanabe et al, 2004). Conversely, peptidoglycan induces a pro-inflammatory phenotype in mutant mice expressing dysfunctional NOD2 (Maeda et al., 2005), suggesting that in some situations, NOD2 mutations might lead to a gain-of-function and elevated pro-inflammatory cytokine production.

Although most commensal bacteria do not activate NF-κB, certain species can restrain inflammatory signals in response to *Salmonella typhimurium* and its flagellin through pathways that seem to involve NF-κB (O'Hara et al., 2006). It has been elucidated several distinct mechanisms by which commensal bacteria limit NF-κB signalling, including inhibition of epithelial proteasome function, degradation of the NF-κB counter-regulatory factor IκBα or nuclear export of the NF-κB subunit, p65, through a peroxisome proliferator-activated receptor (PPAR)γ-dependent pathway (Kelly et al., 2004; Neish et al., 2000; Petrof et al., 2004). Some commensal bacteria might inhibit specific signalling via TLR4 by elevating PPARγ expression and uncoupling NF-κB-dependent target genes in a negative-feedback loop (Dubuquoy et al., 2003).

Responses to commensals and pathogens also may be distinctly different within the mucosal and systemic immune systems. For example, commensals such as *Bifidobacterium infantis* and *Faecalibacterium prausnitzii* have been shown to differentially induce regulatory T cells that results in the production of the anti-inflammatory cytokine IL-10 (O'Mahony et al., 2008), Other commensals may promote the development of T-helper cells, including Th17 cells, which produces a controlled inflammatory response that is protective against pathogens in part, at least, through the production of IL-17 (Lee et al., 2010). The induction of a low-grade inflammatory response, termed as physiologic inflammation, by commensals could be seen to prime the host's immune system to deal more aggressively with the arrival of a pathogen (Pagnini et al., 2010). In addition to all these mechanisms, which clearly contribute to the critical role attributed to gut microbiota in protecting the host from colonization by pathogenic species (Shanahan, 2002), some intestinal bacteria produce a variety of substances, ranging from relatively nonspecific fatty acids and peroxides to highly specific bacteriocins, which can inhibit or kill other potentially pathogenic bacteria, while certain strains produce proteases capable of denaturing bacterial toxins

(Castagliuolo et al., 1999; Corr et al., 2007; O'Hara & Shanahan, 2007; Rea et al., 2010).

It is evident that the immunologic interactions between the microbiota and the host have been studied in great detail; however, the microbiota metabolic functions are also well known (Claesson & O'Toole, 2010; Fraher, O'Toole, & Quigley, 2012; Ley, 2010; Saulnier et al., 2011). Thus, it has been reported the ability of bacterial disaccharidases to salvage unabsorbed dietary sugars, such as lactose and alcohols, and convert them into short-chain fatty acids (SCFAs) that are used as an energy source by the colonic mucosa (Figure 3). Furthermore, SCFAs promote the growth of intestinal epithelial cells and control their proliferation and differentiation (Jones et al., 2008). Also, it has been described that enteric bacteria can produce nutrients and vitamins, such as folate and vitamin K, as well as deconjugate bile salts (Jones et al., 2008) and metabolize some medications within the intestinal lumen, thereby releasing their active fractions. However, the full metabolic potential of the microbiome is far to be completely elucidated, and the potential contributions of the microbiota to the metabolic status of the host in health and disease are of unlimited interest. The application of genomics, metabolomics and transcriptomics can now reveal, in great detail, the metabolic potential of a given organism (Claesson & O'Toole, 2010; Fraher et al., 2012; Saulnier et al., 2011). More recently, it has been reported that microbiota can influence the development and function of the central nervous system, by producing chemicals, including neurotransmitters and neuromodulators, thereby leading to the concept of the microbiota-gut-brain axis (Bravo, Dinan, & Cryan, 2011; Cryan & O'Mahony, 2011; Fleshner, Maier, Lyons, & Raskind, 2011; Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011).

Considering all the above, it is more and more evident that the normal gut microbiota is an essential factor in health, and now it is beginning to understand the impact that the disruption in the microbiota composition, and the subsequent modifications of the interaction microbiota-host, may have on the human being. Some of these consequences are clearly known since long time ago. This is the situation after antibiotic treatment, when different bacterial groups are eliminated or decreased in number, thus allowing the development of other microorganisms that may be pathogenic (Guarner & Malagelada, 2003; Sekirov & Finlay, 2009; Shanahan, 2002). In other situations, when gut motility and/or gastric acid secretion is impaired, it may result in the establishment of a beneficial environment in the small intestine for the proliferation of bacteria that are normally bounded to the colon, thus generating the syndrome knows as small intestinal bacterial overgrowth (SIBO).

In addition, changes in the microbiota composition have been also associated with the pathogenesis of obesity. In fact, the intestinal microbiota can be identified as an active "organ" that is involved in different processes such as (i) the improvement of nutrient bioavailability and degradation of non-digestible dietary compounds, (ii) the supply of new nutrients, and (iii) the removal of harmful, toxic and non-nutritional compounds. These metabolic functions have important implications in human health and nutrition, although they depend on the composition of the microbiota and its complex interactions with the diet and the host (Claesson & O'Toole, 2010; Fraher et al., 2012; Lesniewska et al., 2006; Ley, 2010; Saulnier et al., 2011). Thus, the shift of the bacterial populations in the gut towards those that act as extractors of absorbable nutrients, which are more easily available for their assimilation by the host, could play an important role in the obesity (Ley, 2010).

Furthermore, an altered microbiota composition may also disturb the immunologic interaction between the bacteria and the host, who, for example, begins to recognize the constituents of the normal microbiota as harmful, instead of natural, and may trigger an inappropriate inflammatory response, which, in turn, may ultimately lead to conditions such as IBD (Guarner & Malagelada, 2003; Sekirov & Finlay, 2009; Shanahan, 2002). Supporting this idea, it has been reported that those strategies targeted to modify the intestinal microbiota composition, including the administration of probiotics or prebiotics, are able to attenuate the inflammatory response in experimental models of IBD (Hart et al., 2005; Isolauri & Salminen, 2005; Jijon et al., 2004; McCarthy et al., 2003; Rachmilewitz et al., 2004; Rioux, Madsen, & Fedorak, 2005; Sheil et al., 2004; Thomas & Versalovic, 2010). In fact, some clinical studies have confirmed the beneficial effects exerted by probiotics, like those administering non-pathogenic *Escherichia coli* Nissle 1917 or the yeast *Saccharomyces boulardii*, revealing their efficacy in maintaining remission in human UC (Borody et al., 2003; Damman, Miller, Surawicz, & Zisman, 2012). In addition, it has been reported that *Faecalibacterium prausnitzii*, a bacteria with anti-inflammatory properties, is less abundant in IBD patients than in healthy individuals (Sokol et al., 2009). The reliable importance of microbiota-host interactions in IBD is further supported by different genetic studies of IBD that have identified several genes involved in bacterial recognition, host-bacteria alliance and the resultant inflammatory cascade as significant in the pathogenesis of these intestinal conditions (Van Limbergen, Philpott, & Griffiths, 2011). When considering a more clinical level, the theoretical role of the microbiota is also supported by the efficacy of antibiotics in IBD, as well as by some preliminary data suggesting that faecal transplantation may be effective in IBD

(Damman et al., 2012; Grehan et al., 2010; Guo et al., 2012). Another more convincing clinical example about the beneficial impact obtained after microbiota modulation is pouchitis, which is considered as an IBD variant that occurs in the neorectum in patients with UC who have undergone a total colectomy and ileo-anal pouch procedure. In these patients, the probiotic mixture VSL#3, composed by eight different strains of lactic acid bacteria (*Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbruekii* subsp. *bulgaricus*, *Bifidobacterium infantis*, *Bifidobacterium breve* and *Bifidobacterium longum*), has proven to be effective in the primary prevention and maintenance of remission of patients with pouchitis, since remission was maintained in 85% of patients treated with VSL#3 compared with 6% of patients receiving placebo (Mimura et al., 2004).

PROBIOTICS

According to the Food and Agriculture Organization of the United Nations and the World Health Organization, probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts (FAO/WHO: 2001). Some of the beneficial effects of probiotic consumption include the improvement of intestinal tract health, by means of regulation of microbiota and stimulation and development of the immune system, the production and/or increased bioavailability of nutrients, and a reduced risk of certain gastrointestinal diseases, like lactose intolerance or diarrhea (Guo et al., 2012; Pelletier, Laure-Boussuge, & Donazzolo, 2001; Zeng et al., 2008). However, generalizations concerning the potential health benefits of probiotics should not be made because probiotic effects tend to be strain specific. Thus, the health benefit attributed to one strain is not necessarily applicable to another strain even within one given species (Williams, 2010).

The consideration of a microorganism as a probiotic implies that the following criteria need to be fulfilled: i) It should be isolated from the same species as its intended host; ii) It should have a demonstrable beneficial effect on the host, iii) It should be non-pathogenic, nontoxic, and free of significant adverse side effects; iv) It should be able to survive through the gastrointestinal tract, v) It should be stable during the intended product shelf life and contain an adequate number of viable cells to confer the health benefit, and vi) It should be compatible with product format to maintain desired sensory properties; and labeled accurately.

In table 2 some of the most common and studied probiotics are listed. They commonly belong to genus *Lactobacillus* and *Bifidobacteria*, also others have been also considered like *Streptococcus* and *E. coli* Nissle. Although most abundant are bacteria, also yeast are present like *Saccharomyces boulardii and* a bacteria mix like VSL#3.

The mechanisms underlying the beneficial effects of probiotics are not completely known but are likely to be multifactorial. Several mechanisms of action have been proposed to explain these beneficial effects: enhancement of the epithelial barrier function, increased adhesion to intestinal mucosa and concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms through the production of antimicrobial substances, and modulation of the immune system (Figure 4).

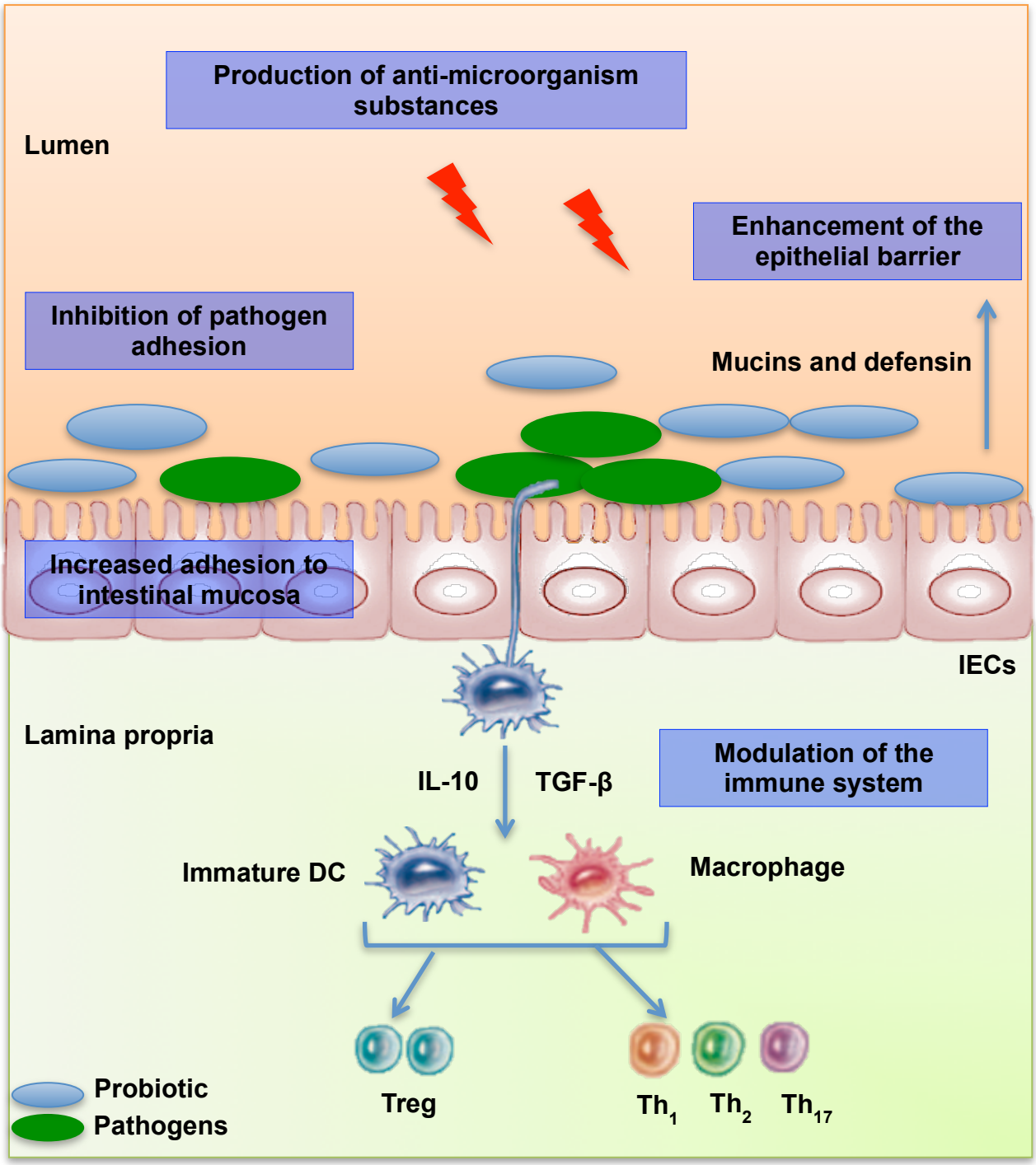


Figure 4. Major mechanisms of action of probiotics.

1. Enhancement of the Epithelial Barrier Function

The intestinal epithelium is in permanent contact with the luminal contents, including the variable and dynamic enteric microbiota, and plays an important role in discriminating the absorbable beneficial nutrients from those products potentially harmful to the human body. Besides, the intestinal epithelium integrity, reinforced by the epithelial junction adhesion complex, is a key component of the intestinal barrier, which constitutes the most important defence mechanism to protect the organism from the environment. Other components that participate in these protective functions are the mucous layer, as well as the released antimicrobial peptides and secretory IgA by different cells located in the intestinal mucosa (Ohland & Macnaughton, 2010). When this barrier function is disrupted, bacterial and food antigens can break through the intestinal mucosa and submucosa, thus facilitating the onset of local inflammatory responses, which may later on result in the development of intestinal disorders like IBD (Hooper, Stappenbeck, Hong, & Gordon, 2003; Hooper et al., 2001; Sartor, 2006).

The mechanisms by which probiotics enhance intestinal barrier function are not fully understood. Some studies have indicated that enhancing the expression of genes involved in TJ signalling is a possible mechanism to strengthen intestinal barrier integrity (Anderson et al., 2010). For instance, in a T84 cell barrier model, lactobacilli modulate the regulation of several genes encoding adherence junction proteins, such as E-cadherin and β-catenin. It has also been reported that lactobacilli differentially influences the phosphorylation of adherence junction proteins and the abundance of protein kinase C (PKC) isoforms, thereby positively modulating epithelial barrier function (Hummel et al., 2012). Other data have reported that probiotics may promote the repair of the barrier function after damage, thus preventing the disruption of the mucosal integrity and restoring it in T84 and Caco-2 cells. This effect is mediated by the enhanced expression and redistribution of TJ proteins of ZO-2 and PKC, resulting in the reconstruction of the TJ complex (Stetinova et al., 2010; Zyrek et al., 2007). Similarly, a recent study has reported that some probiotics protect the epithelial barrier and increases TJ protein expression in vivo and in vitro by activating the p38 and extracellular regulated kinase signalling pathways (Dai, Zhao, & Jiang, 2012).

Furthermore, in different intestinal conditions including human IBD, it has been proposed the existence of a link between altered levels of pro-inflammatory cytokines and increased intestinal permeability (Bruewer, Samarin, & Nusrat, 2006; Sartor, 2006). In this setting, the administration of probiotics that reduce the up-regulated expression and production of cytokines can prevent cytokine-induced epithelial

damage, thus contributing to the reinforcement of the mucosal barrier. Supporting this, it has been reported that two different peptides produced and secreted by *Lactobacillus rhamnosus* GG (LGG), named p40 and p75, are able to prevent cytokine-induced cell apoptosis by activating the anti-apoptotic protein kinase B (PKB/Akt) in a phosphatidyl inositol-3-kinase-dependent pathway and by inhibiting the pro-apoptotic p38/mitogen-activated protein kinase (MAPK) (Yan et al., 2007; Yan & Polk, 2002). The evidence that p40 and p75 are responsible for the observed effects is derived from the observation that the anti-apoptotic function is abolished when p40- and p75-specific antibodies are added in vitro to murine and human epithelial cells or to colon explants derived from mice (Yan et al., 2007).

Finally, mucin glycoproteins, or mucins, are the major macromolecular constituents of the epithelial mucus, and they have long been implicated in health and disease. IECs secrete mucins, which are able to prevent the adhesion of pathogenic organisms (Collado et al., 2005; Gonzalez-Rodriguez et al., 2012). Additionally, in the mucous there are present lipids, free proteins, Igs and salts (Forstner, 1978). Probiotics may promote mucous secretion, which is considerd as an additional mechanism to improve barrier function and facilitate the exclusion of pathogens. The probiotic mixture VSL#3 has been reported to increase the expression of MUC2, MUC3 and MUC5AC in the human intestinal cell line HT29 (Otte & Podolsky, 2004). However, in vivo studies have been shown to be less consistent, since mice given VSL#3 daily for 14 days did not exhibit altered mucin expression or modifications in mucous layer thickness (Gaudier et al., 2005). Conversely, rats given VSL#3 at a similar daily dose for 7 days have a 60-fold increase in MUC2 expression and a concomitant increase in mucin secretion (Caballero-Franco, Keller, De Simone, & Chadee, 2007). Therefore, mucous production may be increased by probiotics in vivo, but further studies are needed to make a conclusive statement.

2. Increased Adhesion to Intestinal Mucosa

The probiotic adhesion to the intestinal mucosa is a prerequisite for colonization, and it is an important characteristic that has been related to the ability of the different strains to modulate the immune system in the host (Beachey, 1981; Juntunen et al., 2001; Schiffrin et al., 1997). Many different intestinal mucosa models have been used to assess the adhesive ability of probiotics, which widely vary depending on the particular strain considered.

It has been proposed that the interaction between probiotic bacteria and host epithelial cells is specific, thus reflecting a possible association between the surface proteins of probiotic bacteria that leads to the competitive exclusion of pathogens from the mucus (Haller et al., 2001; Ouwehand, Salminen, & Isolauri, 2002; Van Tassell & Miller, 2011). Thus, lactobacilli and bifidobacteria have been reported to produce surface proteins, like the adhesins, which mediate attachment to the mucous layer (Buck, Altermann, Svingerud, & Klaenhammer, 2005; Van Tassell & Miller, 2011). The most studied example of mucus-targeting bacterial adhesin is mucus-binding protein (MUB) produced by *Lactobacillus reuteri* (Buck et al., 2005; Hynonen, Westerlund-Wikstrom, Palva, & Korhonen, 2002). The involvement of surface proteins in the interaction with enterocytes has been also reported for *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum*. Under certain circumstances, these proteins may play a role in facilitating the colonization of the human gut through degradation of the extracellular matrix of cells or by facilitating close contact with the epithelium (Candela et al., 2007; Candela et al., 2009; Candela et al., 2011; Guglielmetti et al., 2008). Other important protein, mucous adhesion-promoting protein (MapA), is involved in the binding of different probiotics to mucus (Ouwehand et al., 2002); this activity can be facilitated by the ability showed by some probiotics to induce MUC2 and MUC3 expressions, as well as to inhibit the adherence of enteropathogenic *Escherichia coli*. These observations indicate that enhanced mucous layers and glycocalyx overlying the intestinal epithelium, as well as the occupation of microbial binding sites, provide protection against invasion by pathogens (Hirano et al., 2003; Voltan et al., 2007). Moreover, the probiotic mixture VSL#3 has been reported to increase the synthesis of cell surface mucins and to modulate mucin gene expression, which is dependent on the adhesion of bacterial cells to the intestinal epithelium (Caballero-Franco et al., 2007).

Several observations have indicated that, in response to the deleterious effects exerted by pathogenic bacteria, the host engages its first line of chemical defence by increasing the production of antimicrobial peptides (AMPs), including α- and β-defensins, cathelicidins, C-type lectins and ribonucleases, in an attempt to prevent the disruption of the intestinal epithelial barrier (Ayabe et al., 2000; O'Neil et al., 1999; Ogushi et al., 2001; Takahashi et al., 2001). Many AMPs display enzyme activities that kill bacteria by carrying out an enzymatic attack on cell wall structures and/or non-enzymatic disruption of the bacterial membrane. For instance, the different enzymes expressed by Paneth cells attack the bacterial membranes; thus, lysozyme hydrolyzes the glycosidic linkage of wall peptidoglycan and phospholipase A2 bacterial membrane

phospholipids (Koprivnjak et al., 2002; Muller, Autenrieth, & Peschel, 2005). Moreover, defensins comprise a major family of membrane-disrupting peptides in vertebrates. The interaction of this protein with bacteria is non-specific and mainly driven by its binding to anionic phospholipid groups of the membrane surface through electrostatic interactions, which creates defensin pores in the bacterial membrane that disrupt its integrity and promote lysis of microorganisms (Kagan, Selsted, Ganz, & Lehrer, 1990). Cathelicidins are usually cationic, α-helical peptides that bind to bacterial membranes through electrostatic interactions and, like the defensins, induce membrane disruption (Bals & Wilson, 2003). It has been reported that different probiotic strains, including *Escherichia coli* Nissle 1917, can also induce the release of these AMPs, including defensins from epithelial cells, thus contributing to stabilize the gut barrier function (Furrie et al., 2005; Mondel et al., 2009).

3. Production of Antimicrobial Substances

Among the proposed mechanisms involved in the health benefits attributed to the probiotics are the production of low molecular weight compounds (<1,000 Da), mainly organic acids, and antibacterial substances termed as bacteriocins (>1,000 Da).

Organic acids, like acetic acid and lactic acid, have a strong inhibitory effect against Gram-negative bacteria. Probiotics have been reported to produce these organic acids, which are the main responsible agents for the inhibitory activity of probiotics against pathogens (Alakomi, Matto, Virkajarvi, & Saarela, 2005; De Keersmaecker et al., 2006; Makras, Falony, Van der Meulen, & De Vuyst, 2006). It has been proposed that this is achieved because the undissociated form of the organic acid passes through the pathogen bacterial cell and, once inside the cytoplasm, becomes the dissociated form that either by the eventual lowering of the intracellular pH or by its intracellular accumulation can lead to the death of the pathogenic bacteria (Kirjavainen, Ouwehand, Isolauri, & Salminen, 1998; Russell & Diez-Gonzalez, 1998).

Many probiotics, including lactic acid bacteria (LAB), have been reported to produce bacteriocins, as well as other small AMPs. The bacteriocins produced by Gram-positive bacteria, like lactacin B from *L. acidophilus*, plantaricin from *L. plantarum* or nisin from *Lactococcus lactis*, have a narrow activity spectrum and act only against closely related bacteria; however, other bacteriocins are also active against food-borne pathogens (Kabore et al., 2012). The common mechanisms of

bacteriocins include the destruction of target cells by pore formation and/or inhibition of cell wall synthesis (Hassan et al., 2012). For example, nisin forms a complex with the ultimate cell wall precursor, lipid II, thereby inhibiting cell wall biosynthesis of spore-forming bacilli mainly; subsequently, the complex aggregates and incorporates peptides to form a pore in the bacterial membrane (Bierbaum & Sahl, 2009). Other studies have revealed that those probiotic strains able to produce bacteriorcins exhibit a competitive advantage within complex microbial environments as a consequence of their associated antimicrobial activity. Thus, bacteriocin production may enable their establishment and promote their prevalence derived from the direct inhibition of pathogen growth within the gastrointestinal tract (O'Shea et al., 2012).

Finally, probiotics have been reported to generate a diverse array of health-promoting fatty acids. Indeed, certain strains of bifidobacteria or lactobacilli located in the intestinal lumen have been shown to produce conjugated linoleic acid (CLA), which is considered as a potent anti-carcinogenic agent (Macouzet, Lee, & Robert, 2009; O'Shea et al., 2012). Moreover, some strains of probiotics produce other metabolites that inhibit the growth of fungi and other species of bacteria (Coloretti et al., 2007). Thus, lactobacilli can produce antifungal substances, such as benzoic acid, methylhydantoin, mevalonolactone, short-chain fatty acids or the cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo(L-Phe-traps-4-OH-L-Pro)(Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999; Prema, Smila, Palavesam, & Immanuel, 2010; Sjogren et al., 2003; Strom, Sjogren et al., 2002).

4. Probiotics and the Immune System

It is well known that probiotics can exert an immunomodulatory effect, mainly related to their ability to interact with different immune cells located in the intestinal tissue, including epithelial cells, DCs, monocytes/macrophages and lymphocytes. The most available host immune cells to interact with probiotics are IECs and DCs, which have an important role in innate and adaptive immunity. Both IECs and DCs can interact with and respond to gut microorganisms through their PPRs, like TLRs (Gomez-Llorente, Munoz, & Gil, 2010; Lebeer et al., 2010).

In mammals, the TLR family includes eleven proteins (TLR1–TLR11), although there is a stop codon in the human TLR11 gene that results in a lack of production of TLR11 in humans. Activation of TLRs occurs after binding of the ligand to extracellular leucine-rich repeats. In humans, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are

outer membrane-associated and primarily respond to bacterial surface-associated PAMPs. TLR3, TLR7, TLR8 and TLR9 are found on the surface of endosomes where they respond primarily to nucleic acid-based PAMPs from viruses and bacteria (Gomez-Llorente et al., 2010). Dimerization of TLRs and the highly conserved toll-IL-1 receptor (TIR) domains leads to the recruitment of adaptor molecules, such as myeloid differentiation primary response protein (MyD88), TIR domain-containing adaptor protein and TIR domain-containing adapter-inducing IFN-γ, to initiate signalling activation. The TLR signalling pathway, except for TLR3, involves the recruitment of MyD88, which activates the MAPK and nuclear NF-κB signalling pathways (Lebeer et al., 2010). Furthermore, TLR-mediated signalling has been shown to control DC maturation by inducing the up-regulation of various maturation markers, such as CD80, CD83 and CD86, as well as the CCR7 chemokine receptor (Bermudez-Brito et al., 2012).

It has been reported that the overall tolerant state observed for commensal and probiotic microorganisms is mediated by the action of TLRs, such as TLR3 and TLR7, on DCs (Gomez-Llorente et al., 2010). In this setting, DCs initiate an appropriate response, such as the differentiation of Th0 to Treg, which has an inhibitory effect on Th1, Th2 and Th17 inflammatory responses. Furthermore, TLR signalling has been also considered as essential to mediate the immunomodulatory effects of probiotics.

Peptidoglycan, the main component of Gram-positive bacteria binds TLR2 and activates the intracellular signal in combination with TLR6. Several studies have demonstrated that TLR2 is necessary for some *Lactobacillus* strains to exert their immunomodulatory effects, thus supporting the key role of TLR signalling in probiotic activity (Shida et al., 2009). In fact, it has been suggested that the intact peptidoglycan of lactobacilli essentially acts via TLR2 to inhibit IL-12 production. However, and although this recognition by TLR2 is essential, between 12 and 48% of IL-12 production in TLR2-deficient macrophages is inhibited by peptidoglycan, thus suggesting that other TLR2-independent mechanisms may also be involved. Similarly, Zeuthen et al. (2008) (Zeuthen, Fink, & Frokiaer, 2008) showed that TLR2–/– DCs produce more IL-2 and less IL-10 in response to bifidobacteria, thus concluding that the inhibitory effect of bifidobacteria on immune response is also dependent on TLR2.

Correspondingly, heat-inactivated LGG and *Lactobacillus delbrueckii* subsp. *bulgaricus* can decrease TLR4 expression, similar to the effect achieved with LPS, after 12 h incubation in human monocyte-derived DCs. Moreover, LGG downregulates p38 expression, and *L. delbrueckii* subsp. *bulgaricus* reduces IκB expression. In

addition, these probiotic strains can modify the immune response at the post-transcriptional level by modifying miRNA expression (Giahi, Aumueller, Elmadfa, & Haslberger, 2012).

TLR9 has been also reported to be crucial for probiotic beneficial effects. This TLR recognizes bacterial CpG DNA and synthetic unmethylated CpG olignucleotide mimics. Unmethylated DNA fragments containing CpG motifs that are released from probiotics in vivo have the potential to mediate anti-inflammatory effects through TLR9 signalling at the epithelial surface. Since the different lactobacilli species differ in their C+G composition, the ability of each probiotic belonging to this group in stimulating TLR9 is likely to be different (Hemmi et al., 2000; Wells, 2011). In vitro studies using polarized HT29 and T84 cell monolayers, Ghadimi et al. (Ghadimi et al., 2011) showed that the binding of natural commensal-origin DNA to the apical TLR9 initiates a specific intracellular signalling cascade, attenuates TNF-α-induced NF-κB activation, and the subsequent NF-κB-mediated IL-8 expression. Similarly, when LGG DNA was apically applied, it was observed a diminished TNF-α-induced NF-κB activation, associated with reduced IκB degradation and p38 MAPK phosphorylation. Furthermore, TLR9 silencing abolishes the inhibitory effect of natural commensal-origin DNA on TNF-α-induced IL-8 secretion.

In addition to TLRs, there is another family of membrane-bound receptors: NLRs. The most thoroughly characterized members are NOD1 and NOD2, although more than 20 NLRs have been reported so far (Hakansson & Molin, 2011). NOD1 can sense peptidoglycan moieties containing mesodiaminopimelic acid, which are associated with Gram-negative bacteria, but NOD2 senses muramyl dipeptide motifs, which can be found in a wide range of bacteria (Biswas et al., 2010). Upon recognition of their agonist, both NOD1 and NOD2 self-oligomerize to recruit and activate the adaptor protein kinase containing a caspase recruitment domain (RICK), a protein kinase that regulates CD95-mediated apoptosis, which is essential for the activation of NF-κB and MAPKs, resulting in the up-regulation of transcription and production of inflammatory mediators (Chen & Pedra, 2010). In fact, there are a few studies showing the effect of probiotics on NLR (Macho Fernandez et al., 2011); demonstrated that the protective capacity of *L. salivarius* Ls33 correlates with local IL-10 production, which is abolished in NOD2-deficient mice. Indeed, these authors showed that the anti-inflammatory effect of Ls33 is mediated via NOD2.

Another key pathway involves the NLRs is the apoptosis-associated speck-like protein with caspase recruitment to activated caspase 1 (an adaptor protein that is

necessary for the cleavage of pro-IL-1β and pro-IL-18 into their mature and biologically active forms). NLRs participate in the formation of inflammasomes, which leads to the activation of caspase-1. There are three principal inflammasomes, concretely NLRP3 detects LPS, MDP, bacterial RNA and viral RNA (Chen & Pedra, 2010). It has been suggested that NLRP3 has an important role in the regulation of human intestinal inflammation, such as in CD (Hirota et al., 2011), and that dysregulated NLRP3 expression results in the disruption of immune homeostasis associated with auto-inflammatory disease in humans (Anderson et al., 2008). It has been found that *Lactobacillus delbrueckii* subsp. *bulgaricus* NIAI B6 and *Lactobacillus gasseri* JCM1131T are able to enhance NLRP3 expression in the gut-associated lymphoid tissue (GALT) of adult and newborn swine. These results have suggested that immunobiotic *Lactobacillus* strains directly promote NLRP3 expression via TLR and NOD-mediated signalling, resulting in the induction of appropriate NLRP3 activation in porcine GALT (Tohno, Shimosato, Aso, & Kitazawa, 2011).

Table 2. The most common and used probiotics.
Lactobacilos- *L.casei, L.paracasei, L.acidophilus, L.rhamnosus GG, L.brevis, L.plantarum, L.delbrueckii, L.gaserii, L.helveticus*
Bifidobacterium- *B.bifidum, B.infantis, B.adolescentis, B.longum, B.breve*
Escherichia coli- *Escherichia coli* Nissle 1917
Streptococcus- *S.salivarius subsp. termophilus*
Saccharomyces- *S.boulardii*
VSL#3 (S.salivarius subsp. termophilus, *L.casei*, *L.plantarum*, *L.delbruckiisubsp. bulgaricus*, *B.infantis*, *B.breve*, *B.longum*)

Lactobacillus fermentum and *Lactobacillus salivarius* in IBD

Lactobacilli are a major component of the gut microbiota of humans and animals, and for this reason they are the most frequently used as probiotics (Ahrne et al., 1998). Lactobacilli are found in the gastrointestinal tract of humans and animals in variable amounts depending on the animal species, age of the host, or location within the gut. The genus *Lactobacillus* comprises a large heterogeneous group of low-G+C gram-positive, nonsporulating, and anaerobic bacteria (Claesson, van Sinderen, & O'Toole, 2007). Taxonomically, the genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli,* order *Lactobacillales*, family *Lactobacillaceae*. These genera can produce organic acids from carbohydrate fermentation, such as LAB and acetic acid, which can interfere with the growth of surrounding microorganisms. In addition, these microorganisms can also produce hydrogen peroxide that is an antimicrobial substance (McGroarty et al., 1992). Finally, it has been demonstrated that some of the bacteria included in these genera possess strong antioxidant potential (Achuthan et al., 2012; Kullisaar et al., 2002).

It is widely accepted the health benefits of lactobacilli, especially for the treatment and prevention of enteric infections and post-antibiotic syndromes (Lebeer, Vanderleyden, & De Keersmaecker, 2008). Meta-analyses have established the efficacy of some lactobacilli in acute infectious diarrhoea and the prevention of antibiotic-associated diarrhoea (Sazawal et al., 2006). Furthermore, their administration may reduce the recurrence of *Clostridium difficile*-associated diarrhoea (Pillai & Nelson, 2008) and prevent necrotizing enterocolitis in preterm neonates (Deshpande, Rao, Patole, & Bulsara, 2010). Some promising results have also been obtained for the prevention and treatment of IBD (Hedin, Whelan, & Lindsay, 2007), prevention of colorectal cancer (Rafter et al., 2007), and treatment of irritable bowel syndrome (IBS) (Camilleri, 2006). Furthermore, there are some evidences that lactobacilli can modify the immune system, showing immuno-modulatory properties. In Pochard's study in 2005 (Pochard et al., 2005), lactobacillus were reported to be able to secrete bioactive IL-12, a critical factor in switching naive or memory T cells to Th1 response (Pochard et al., 2005). In agreement with this result, other report showed that different strains of lactobacilli induce low levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-8), while inducing high levels of IFN-γ and IL-12p70 (Perdigon et al., 2002; Sun et al., 2013). Moreover, it has been reported that lactobacilli activate innate immune cells such as APCs via PRRs and induce the secretion of cytokines that influence the polarization of activated T cells (Mohamadzadeh et al., 2005; Ou, Lin, Tsai, & Lin, 2011).

Particularly, *Lactobacillus fermentum* and *Lactobacillus salivarius* are two important examples in this group of probiotics, and they have long been studied because of its human origin and adherence capacity to the gastrointestinal tract, as well as their antagonistic growth properties against different pathogens (Orrhage & Nord, 2000).

As commented above, different strains from a given probiotic species can show a different pattern of biological activities and, in consequence, the results of one specific *Lactobacillus* strain cannot be generalized to others. Of course, this is also true for the reported intestinal anti-inflammatory effects of probiotics, which have been mainly evidenced in experimental models in rodents.

In this regard, it has been described that *L. fermentum* Lf1 was quite effective in reducing the severity of DSS-colitis in mice (Chauhan et al., 2014). In this assay, *L. fermentum* Lf1 proved to have multifactorial antioxidative and anti-inflammatory defence arsenal, not only to protect its own survival but also to confer protection to the host cells against the hostile oxidative stress confronted in the mice gut during colitis.

L. fermentum BR11 also showed intestinal anti-inflammatory effect in the DSS model of rat colitis, when evaluated macroscopically, by attenuating the colitic symptoms in this model, including weight loss, blood in faeces and diarrhoea, and ameliorating the shortening of the colon length that characterized the intestinal inflammation, as well as microscopically, since it prevented the distal colon crypt hyperplasia (Geier et al., 2007).

The pre-treatment with *L. fermentum* CECT5716 resulted in an amelioration of the inflammatory response in the TNBS model in rat colitis. These beneficial effects were evidenced histologically with an improvement of the architecture of the inflamed tissue, and it was associated with increased levels of glutathione, thus revealing an antioxidant mechanism, as well as with inhibition in the production of some of the inflammatory mediators, such as TNF-α and nitric oxide, involved in the intestinal inflammatory response (Peran et al., 2006). In addition, the treatmentwith *L. fermentum* CECT5716 was able to decrease the cyclo-oxygenase-2 (COX-2) expression, and promoted the growth of Lactobacilli species in comparison with control colitic rats, and increased the production of SCFA in the colonic contents (Peran et al., 2007; Peran et al., 2007). The beneficial effects exerted by this strain were confirmed in the TNBS model of colitis in mice, being able to reduce the histological score and to decrease IL-6 production and increased MyD88 staining (Mane et al., 2009).

Other strain of *L. fermentum*, ACA-DC 179, has been also reported to show a preventative role in TNBS-induced colitis and *Salmonella*-infection induced in mice.

These beneficial effects were attributed to its antimicrobial activity and immunomodulatory properties (Zoumpopoulou et al., 2008).

Besides, *Lactobacillus salivarius* is part of the indigenous microbiota of the gastrointestinal tract and oral cavity of humans and other animals. Many studies have demonstrated the properties of this probiotic strain and its utility (Corr et al., 2007; Messaoudi et al., 2012; Messaoudi et al., 2013; Neville & O'Toole, 2010). In vitro assays in HT-29 cells have showed that *Lactobacillus salivarius* subsp. *salivarius* UCC118 functionally modulated the epithelium by attenuating *Salmonella typhimurium*-induced NF-κB activation and IL-8 secretion (O'Hara et al., 2006). Furthermore, Corr and collaborators (2007) (Corr et al., 2007) have shown that this strain protects against *Listeria monocytogenes* EGDe and LO28 and *Salmonella typhimurium* UK1 infections in mice (Corr et al., 2007). Otherwise, this strain has anti-infective activity due to production of the bacteriocin Abp118, a broad-spectrum class IIb bacteriocin, which may impact the microbiota. Surprisingly, the results in different animal models revealed an effect on Gram-negative microorganisms by *L. salivarius* UCC118 administration and production of Abp118, even though Abp118 is normally not active in vitro against this group of microorganisms (Riboulet-Bisson et al., 2012). Nowadays much attention has been focused on the direct anti-inflammatory capacities of lactobacilli on tolerogenic DCs, the generation of regulatory T cells in vivo and in vitro, and the induction of IL10 in in vitro assays (Kwon et al., 2010). However, there are also reports that demonstrate that Lactobacillus-induced suppression of pro-inflammatory immune responses, independent of IL10 or regulatory T cells (Foligne et al., 2007; Niers et al., 2005; Schultz et al., 2002; Xia et al., 2011). To date, it remains largely unknown how lactobacilli affect the balance between pro- and anti-inflammatory immune cell populations in vivo. In fact, *L. salivarius* UCC118 treatment showed a clear reduction of Th2 responsiveness combined with only a modest increase in $CD8^+$ T cell responsiveness (Smelt et al., 2012).

L. salivarius ssp. *salivarius* CECT5713 treatment in the TNBS model in rats facilited the recovery of the inflamed tissue, ameliorating the production of some mediators involved in the inflammatory response, such as TNF-α and nitric oxide (Peran et al., 2005). Additionally, in a more recent study, Sierra and collaborators (2010) showed that the administration of *L. salivarius* CECT5713 improved host immunity by inducing IL-10 and some immunoglobulin levels as well as inducing an increase in NK cell and monocyte numbers (Sierra et al., 2010).

Taken together, the application of probiotic lactobacilli in IBD assumes that the mechanisms underlying the health-promoting capacities belong to one of the following; (i) pathogen inhibition and restoration of microbial homeostasis through microbe-

microbe interactions, (ii) enhancement of epithelial barrier function, and (iii) modulation of immune responses (gradually, lactobacilli have been investigated for their capacities to exert immunostimulatory (adjuvant) and immunoregulatory properties). Although, given the complexity of these three main functions, it can be understood that different strains evoke different responses in the host.

Saccharomyces boulardii in IBD

Saccharomyces boulardii is a thermophilic nonpathogenic yeast that is selectively used for prophylaxis and treatment of antibiotic-associated and traveller's diarrhoea (Surawicz et al., 1989). *Saccharomyces boulardii* belongs to the group of simple eukaryotic cells (such as fungi and algae) and thus differs from bacterial probiotics that are prokaryotes. More recently, clinical as well as experimental evidence in mouse models of inflammation suggest that this probiotic yeast may have a therapeutic potential for IBD patients. Importantly, *S. boulardii*, has demonstrated clinical and experimental effectiveness in gastrointestinal diseases with a predominant inflammatory component, indicating that this probiotic might interfere with cellular signalling pathways common in many inflammatory conditions. One study performed in peripheral blood mononuclear cells (PBMC) suggest that *S. boulardii* may exhibit an anti-inflammatory effect through modulation of DCs phenotype, function and migration by inhibition of their immune response to bacterial microbial surrogate antigens such as LPS (Thomas et al., 2009). The main mechanisms of action of *S. boulardii* include antimicrobial activities, trophic effects upon the intestinal mucosa, and the modification of the host-signalling pathways that are involved in inflammatory and non-inflammatory intestinal diseases. It has been shown that *S. boulardii* inhibits the production of pro-inflammatory cytokines by inhibiting the main regulators of inflammation, such as NF-κB and mitogen-activated protein kinases, which play crucial roles in the pathogenesis of IBD (Coskun, Olsen, Seidelin, & Nielsen, 2011; Sougioultzis et al., 2006).

In fact, several clinical trials have indicated that *S. boulardii* might be beneficial as an adjunctive therapy in CD as well as flare-ups in UC patients (Guslandi, Giollo, & Testoni, 2003; Guslandi, Mezzi, Sorghi, & Testoni, 2000; Plein & Hotz, 1993). In addition, studies with animal IBD models underline the potential for *S. boulardii* to reduce inflammatory colonic responses and provide potential mechanisms involved in this beneficial effect. Using the TNBS-induced colitis model, Lee and colleagues (2009) found that *S. boulardii* whole yeast administration substantially reduced all

aspects of colitis, including histological damage, diarrhoea, and mucosal levels of the pro-inflammatory mediators IL-1β, IL-6, TNF-α, and inducible nitric oxide synthase (iNOS) (Lee et al., 2009). Furthermore, the same group of investigators showed that cultures of *S. boulardii* stimulated the expression of PPAR-γ, nuclear receptor expressed in the colon and colonic epithelial cells, and represents a novel therapeutic target in intestinal inflammation and IBD (Dubuquoy et al., 2006) in HT-29 cells (Lee et al., 2005) and elevated the transcription of this receptor in the colon of animals exposed to TNBS. Interestingly, silencing of PPAR-γ expression in colonocytes reverses the inhibitory effect of *S. boulardii* in IL-8 gene expression, suggesting that activation of PPAR-γ in response to *S. boulardii* represents another molecular mechanism involved in its anti-inflammatory action (Lee et al., 2009).

Similarly, the *S. boulardii* pre-treatment in DSS model reduced clinical score and severity of colitis and decreased colonization of *Candida albicans* following DSS administration (Jawhara & Poulain, 2007). Although the mechanisms of these beneficial effects are not clear, the authors of this study provided evidence that TLRs might play a role in this response.

Nevertheless, an exciting study by Dalmasso et al. (Dalmasso et al., 2006) has provided evidences for a novel *S. boulardii* mechanism involved in IBD colitis. These investigators examined the effect of *S. boulardii* administration in a T cell transfer model of colitis in severe combined immuno-deficient (SCID) mice and found that the probiotic yeast prevents colonic inflammation and clinical signs of colitis (Dalmasso et al., 2006). These protective responses are associated with diminished colonic NF-κB activity and reduced levels of several pro-inflammatory cytokines. In this study, the yeast treatment reduced IFN-γ production by CD4+ T cells in the colon but increased it in the mesenteric lymph nodes, indicating a possible redistribution of IFN-γ–producing T cells. Additional transfer experiments demonstrated that increased accumulation of CD4+ T cells takes place when the receiving, but not the donor mice are provided with *S. boulardii*, indicating that T-cell retention occurs at the mesenteric lymphatic tissue. Furthermore, it was identified a soluble factor in *S. boulardii* conditioned media involved in endothelial cell-mediated rolling of T-cells (Dalmasso et al., 2006), although the identity of this factor(s) remains to be elucidated.

There have been reported several studies based on the intestinal inflammatory phenotype observed in *Citrobacter rodentium* infection and its important role in the pathogenesis of IBD (Hooper et al., 2001). Several groups use *C. rodentium* mouse infection as an experimental model to study IBD (Eckmann, 2006). In this model *S. boulardii* administration has showed a reduced colonic inflammation, a reduced weight loss and an inhibited histological damage (Wu et al., 2008). These ameliorating

effects of *S. boulardii* are associated with significantly lower numbers of *C. rodentium* adherent to the mucosa, as well as reductions in Tir protein secretion and translocation into mouse colonocytes, and expression and secretion of EspB (Wu et al., 2008), both important virulent factors (Frankel et al., 2001). Although *S. boulardii* has no direct bactericidal effect against *C. rodentium*, this probiotic is able to reduce the expression of EspB and Tir proteins in vitro (Wu et al., 2008). Particularly, *S. boulardii* provides protection against colitis associated with *C. rodentium* infection by releasing soluble factor(s) able to modulate *C. rodentium* adherence to epithelial cells and inhibiting expression of potent virulent factors secreted by this microbe.

There are increasing evidences from experimental mouse models and from clinical observations that angiogenesis is an important component of IBD pathogenesis (Danese, 2008; Papa et al., 2008). It has also been reported in experimental colitis that vascular endothelial growth factor (VEGF) is an important mediator of IBD through promoting intestinal angiogenesis and inflammation (Scaldaferri et al., 2009). Over-expression of VEGF in mice with DSS-induced colitis worsened their condition, whereas over-expression of soluble VEGFR, to block VEGF effects, presented a beneficial effect (Scaldaferri et al., 2009). Recently, Chen et al. (2013) (Chen et al., 2013) have reported that S.boulardii blocks VEGFR signalling and inhibits angiogenesis both in vitro and in vivo (Chen et al., 2013).

Escherichia coli Nissle 1917 and IBD

Escherichia coli strain Nissle 1917 (EcN) is the active component of the microbial drug Mutaflor (Ardeypharm GmbH, Herdecke, Germany). This strain is used in several European countries as a probiotic drug for the treatment of IBD (Schultz & Lindstrom, 2008). Although *E. coli* Nissle was originally isolated in 1917, the underlying mechanism of its beneficial effect in various intestinal diseases, including ulcerative colitis still remains elusive. A number of studies have shown positive results when this strain is used in conditions such as CD, pouchitis, IBS or necrotizing enterocolitis (Yan & Polk, 2010); but it is especially used in the prevention of relapse in patients with UC. In fact, some clinical trials have demonstrated the efficacy of Nissle 1917 for the UC treatment (Kruis & Schreiber, 2004; Kruis et al., 1997; Rembacken et al., 1999). Double blind randomized controlled trials comparing the efficacy of EcN to that of mesalazine, which is commonly used for treating IBD, have shown that EcN is as efficient as mesalazine to prevent relapse of UC. In addition, the probiotic treatment is associated with a prolonged remission without any reported adverse effects. Studies

performed in vitro in IECs have suggested that EcN suppresses TNF-α-induced IL-8 secretion. Moreover, the mechanism by which EcN suppresses IL-8 might be independent of the inhibition of the NF-κB signalling pathway (Kamada et al., 2008).

Recently, it was shown in the streptomycin treated mouse model that EcN can limit the growth of pathogenic *E. coli* O157 when administrated as treatment in precolonized mice (Leatham et al., 2009).

Although EcN is known to produce microcins M and H47, this does not seem to be efficient in eradicating the IBD associated *E. coli* (Patzer et al., 2003). This does not rule out that EcN in the human intestine interact with the possible harmful IBD associated *E. coli* by blocking their attachment to epithelial cells. In fact, in vitro experiments with an intestinal cell line support the EcN's ability to block the adherence of AIEC (Boudeau et al., 2003).

Different assays have suggested that EcN induces human ß-defensin 2 (*hBD-2*) expression in the cell culture in a time and density dependent manner (Wehkamp et al., 2004).

In addition, EcN can modulate intestinal immune function. Of note, this microorganism possesses a specific LPS that renders it immunogenic, without showing any immunotoxic properties (Grozdanov et al., 2002). Furthermore, this probiotic strain may down-regulate the expansion of newly recruited T cells into the mucosa and limit intestinal inflammation, without affecting already activated lamina propria T cells, thus preserving their role in eliminating deleterious antigens in order to maintain immunological homeostasis, which is clearly beneficial in the treatment of IBD (Sturm et al., 2005). Furthermore, EcN has been shown a secretion of pro-inflammatory cytokines decreased, IL-2, TNF-α and IFN-γ, and increasing in the secretion of anti-inflammatory cytokines, like IL-10 (Sturm et al., 2005). Also, it has been reported that this *Escherichia* strain ameliorated DSS-colitis and decreased proinflammatory cytokine secretion. In fact, in TLR-2 knockout mice a selective reduction of IFN-γ secretion was observed after EcN treatment. Furthermore, co-culture of EcN and human T cells increased TLR-2 and TLR-4 protein expression in T cells and increased NF-κB activity via TLR-2 and TLR-4. Thus, EcN ameliorates experimental induced colitis in mice via TLR-2- and TLR-4-dependent pathways (Grabig et al., 2006). Furthermore, this probiotic strain may reinforce the mucosal barrier and restore it when it is disrupted, specifically through the up-regulation of expression of the mRNA for the proteins ZO-1 and ZO-2 in intestinal epithelial cells, thereby reducing intestinal permeability (Ukena et al., 2007; Zyrek et al., 2007).

Similarly, EcN has revealed to have anti-inflammatory effects both TNBS-induced colitis in rats and LPS-induced organ damage in mice (Arribas et al., 2009).

It is important to emphasise an important global gene regulatory mechanism, which is used by gram-negative as well as gram-positive bacteria, enabling individual bacteria to communicate and coordinate their behaviour in populations. In general terms, it is often defined as cell density-dependent regulation of gene expression via extracellular signals. This communication of bacteria with each other is termed "quorum sensing" (QS) (Fuqua, Winans, & Greenberg, 1994) and it is produced by small, diffusible signals, named "autoinducers". Autoinducer (AI-2) is responsible for the interspecies communication (Surette, Miller, & Bassler, 1999). Jacobi CA et al (2012) showed, for the first time, that AI-2 molecules are produced by EcN in a density dependent manner. AI-2 affects the regulation of cytokine expression in the DSS mouse model. The mutant mice (do not produced AI-2) showed a higher expression of pro-inflammatory cytokines, but a reduced expression of the anti-inflammatory cytokine IL-10 or the mBD-1. Thus, it remains to be seen if AI-2 is influencing the probiotic properties of this important bacterium (Jacobi et al., 2012).

Studies performed in knockout mouse models (129/SvEv) have shown that chronic gut inflammation in IL-10−/− mice results in a reduction of gut microbiota diversity and a strong increase in intestinal E. coli (Wohlgemuth, Haller, Blaut, & Loh, 2009). The increase in the number of *E. coli* in the inflammatory tissue is most probably related to the abundance of iron ions available for siderophores produced by the bacteria (Law, Wilkie, Freeman, & Gould, 1992). A recent study has demonstrated that the increase in the numbers of *E. coli* in the inflammatory tissues is related to the presence of chuA and iutA genes, which facilitate iron acquisition during chronic intestinal inflammatory processes (Pilarczyk-Zurek et al., 2013).

EcN has been shown to regulate the intestinal epithelial cell differentiation factors hairy and enhancer of split-1 (Hes1), atonal homolog 1 (Hath1), and Kruppel-like factor 4 (KLF4), Muc1 and hBD-2 in mice, and in the LS174T colon adenocarcinoma cell line (Becker et al., 2013). Moreover, EcN co-cultured with Caco-2 and mucin-producing LS-174T cells antagonized the activity of some strains of enterohemorrhagic *Escherichia coli* (EHEC), which is responsible for the hemolytic uremic syndrome (Rund, Rohde, Sonnenborn, & Oelschlaeger, 2013). In addition, EcN has demonstrated anti-pathogenic properties against D-associated *E. coli* LF829 and its flagellum has been reported to play a pivotal role in competition against other pathogens (Troge et al., 2012).

Finally, the potential value of factors derived from EcN has been investigated to a far lesser extent. Supernatant from EcN partially protected the small intestine from 5-

fluorouracil (5-FU)-induced damage in rats (Prisciandaro et al., 2011) and in IEC-6 cells when grown in tryptone soya broth (TSB) (Prisciandaro et al., 2012). Besides, although the underlying mechanisms of the protective effects of this EcN supernatant are not well defined, the supernatant of EcN grown in Standard-I-Bouillon growth medium has been shown promising in the treatment of human gastrointestinal motility disorders (Bar et al., 2009). Wang and collaborators (2014) evaluated viability, apoptosis, and monolayer permeability in IEC-6 cells in the presence and absence of the antimetabolite chemotherapy drug, 5-FU. Also, it has been determined if EcN cultured in a range of different growth media resulted in the release of factors that would differentially impact on these parameters. The results revealed that total protein content significantly increased in all EcN supernatants, which confirmed that at least, some of the released factors derived from EcN were proteinaceous in nature. In addition, the proportion of viable cells measure by flow cytometry in the presence of 5-FU was increased and late-apoptotic cells were reduced after 24 and 48 h, compared with 5-FU control. Moreover, all EcN supernatants significantly reduced the disruption of IEC-6 cell barrier function induced by 5-FU, compared with Dulbecco's Modified Eagle's medium (DMEM) control. Thus it has been demonstrated that EcN derived factors could potentially reduce the severity of intestinal mucositis (Wang et al., 2014).

Objectives

OBJECTIVES

The term IBD comprises two related pathologies: Crohn's disease and ulcerative colitis. Both conditions are characterized by chronic inflammation of the intestine, most probably due to an exacerbated immune response in the intestine against an antigen which has not been determined yet, in which the alternation of periods of exacerbation and remission of symptoms takes place. At present there is no ideal treatment that combines efficacy and absence of adverse effects, and, in consequence, it is attractive the development of new strategies that combine efficacy and safety. At present, there are many studies that describe the potential use of probiotics, in the treatment of IBD, through modulation of the intestine microbiota, although the results obtained cannot clearly establish the beneficial effect in all the studies. Recently, it has been reported the key role that miRNAs may play in those conditions associated with an altered immune response, including IBD. Different studies revealed that the intestine microbiota composition is modified in human IBD (dysbiosis), and this seems to be crucial in the development of these intestine conditions. In consequence, it would be interesting to study the possible existence of a relationship among intestine microbiota, microRNA expression profile and altered immune response. With this purpose, in the present Thesis we will use two experimental models of mice colitis: the DNBS and DSS, which are largely used in the preclinical assays for the study of new treatments potentially applicable to human IBD.

The main objectives are:

1) To establish the relationship among modification in the intestine microbiota, miRNA expression profile and development of intestine inflammation in these two experimental models of rodent colitis.

2) To evaluate whether the intestine anti-inflammatory effect showed by different probiotics are related with a modification in the intestine microbiota, and if the modulation of the intestinal immune response can be associated with a modification in the altered miRNA expression observed in the intestinal inflammatory process. In addition, the changes in the mRNA expression in cell types involved in the immune response (epithelial cells and macrophages) will be studied.

Finally, the viability of probiotics has been considered as a requisite to exert their beneficial effect, although some recent studies have reported that some probiotics are able to show intestinal anti-inflammatory effects in experimental model of colitis. In this Thesis, we will check if the viability of one of the probiotics studied, Lactobacillus fermentum, is essential for displaying its properties in these intestinal conditions.

All these results would help to have a more complete characterization of probiotic-based therapeutic strategies as potential treatments to be used in human IBD.

Materials & Methods

MATERIALS & METHODS

PREPARATION OF THE PROBIOTICS

The probiotics used were two of the Lactobacillus genus provided by Biosearch, S.A. (Granada, Spain): *Lactobacillus fermentum* CECT5716, a human breast milk derived strain and *Lactobacillus salivarius* CECT5713 (Olivares, Diaz-Ropero, Martin, Rodriguez, & Xaus, 2006); *Escherichia coli* Nissle 1917 provided by Ardeypharm GmbH (Herdecke, Germany); and *Saccharomyces boulardii* CNCMI-745, yeast provided by Biocodex (Beauvais, France). All of them were normally grown in MRS media at 37°C under anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, UK). For probiotic treatment, bacteria were prepared daily after their suspension in sterile phosphate-buffered saline (PBS) solution. Dead bacteria were obtained after heating the microorganisms at 95ºC for 30 minutes.

EVALUATION OF THE INTESTINAL ANTI-INFLAMMATORY EFFECTS OF THE PROBIOTICS: IMPACT ON miRNA EXPRESSION AND INTESTINAL MICROBIOTA COMPOSITION

IN VIVO STUDIES

All the studies were carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health. All the animals were housed in makrolon cages, maintained in an air-conditioned atmosphere with a 12h light-dark cycle, and they were provided with free access to tap water and food.

Dextran sodium sulfate model of mouse colitis.

Male C57BL/6J mice (7-9 weeks old; approximately 20 g) obtained from Janvier (St Berthevin Cedex, France) were randomly assigned to six different groups of 10 animals each: non-colitic and DSS colitic group received orally PBS solution (200μl) and four groups were treated with the different probiotics. All probiotics were administered orally at the concentration of $5x10^8$ colony-forming unit (CFU), except *Sacharomyces boulardii* that was given at $5x10^9$ CFU, suspended in 200μl of PBS, by

means of an oesophageal catheter, daily for 26 days. Two weeks after starting the experiment, the colitis was induced by adding DSS (36-50 KDa, MP Biomedicals, Ontario, USA) in the drinking water at the concentration of 3% for a period of 6 days, after which DSS was removed (Mahler et al., 1998) (Figure 5). Mice from the non-colitic group were administered PBS solution during the whole experiment. All mice were sacrificed 26 days after the beginning of the experiment.

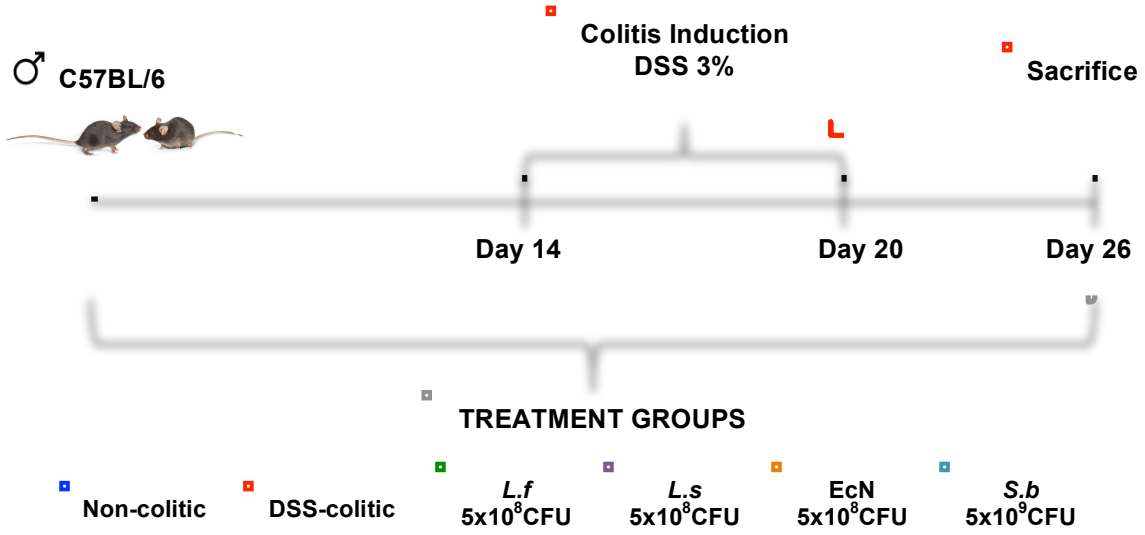


Figure 5. Experimental design in the DSS model of mouse colitis.

L.f (*Lactobacillus fermentum* CECT5716), *L.s* (*Lactobacillus salivarius* CECT5713), EcN (*Escherichia coli* Nissle 1917), *S.b* (*Saccharomyces boulardii* CNCMI-745)

Animal body weight, the presence of gross blood in the faeces and stool consistency were evaluated daily for each mouse by an observer unaware of the treatment. These parameters were each assigned a score according to the criteria proposed previously by Cooper et al. (1993) (Cooper, Murthy, Shah, & Sedergran, 1993) (Table 3) and used to calculate an average daily disease activity index (DAI). Once the animals were sacrificed, the colon was removed aseptically and weighed, and its length was measured under a constant load (2 g). Representative whole colonic tissue was subsequently sectioned in small fragments for biochemical determinations and RNA/miRNA isolation. Finally the faecal content was taken from each animal to do genomic DNA extraction. Then, amplification of 16S rRNA gene V1-V3 region by polymerase chain reaction (PCR) and pyrosequencing of amplicons were performed as described previously (Zhang et al., 2010).

Score	Weight loss	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1- 5%		
2	5- 10 %	Loose stools	
3	10 – 20 %		
4	> 20 %	Diarrhoea	Gross bleeding

Table 3. Scoring of disease activity index (DAI). DAI value is the combined scores of weight loss, stool consistency, and rectal bleeding divided by 3. Adapted from Cooper et al. (1993) (Cooper et al., 1993).

Dinitrobenzene sulphonic acid model in mice.

Adult male CD1 mice (6-8 weeks old, weighing 20–25 g) (Janvier, male CD1 mice). All animals received standard pelleted chow and tap water ad libitum. Mice were randomly allocated in 6 groups of ten animals. Non-colitic and colitic groups received orally PBS solution (200μl) and the other four groups were treated with the different probiotics. All probiotics were administered orally at the concentration of $5x10^8$ CFU, except *Sacharomyces boulardii* that was given at $5x10^9$ CFU, suspended in 200μl of PBS, by means of an oesophageal catheter, daily for 24 days. The colitis was induced twenty days after starting the experiment with DNBS (3mg/mouse) instilled rectally and suspended in 50% ethanol. All animals were sacrificed after 4 days (Figure 6).

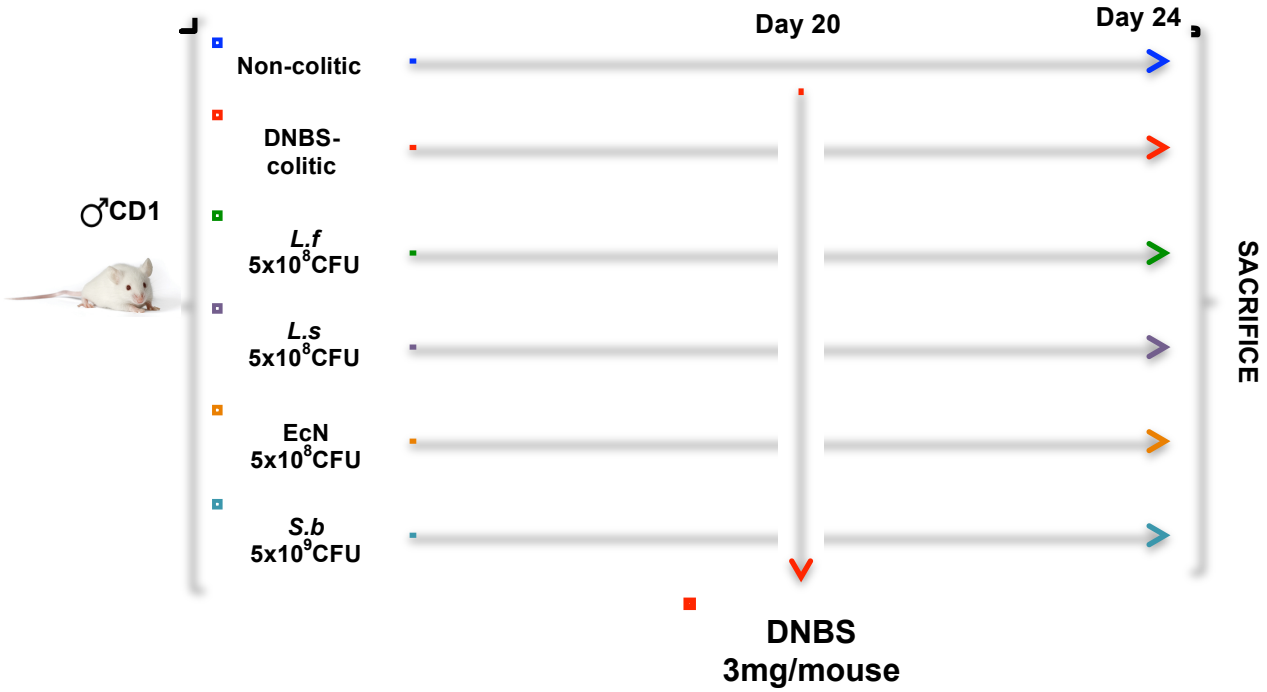


Figure 6 . Experimental design in the DNBS model of mouse colitis.

L.f (*Lactobacillus fermentum* CECT5716), *L.s* (*Lactobacillus salivariussalivarius* CECT5713), EcN (*Escherichia coli* Nissle 1917), *S.b* (*Saccharomyces boulardii* CNCMI-745)

Animal body weight, the presence of gross blood in the faeces and stool consistency were evaluated daily for each mouse by an observer unaware of the treatment. Once the animals were sacrificed, the colon was removed aseptically and weighed, and its length was measured under a constant load (2 g). Representative whole colonic tissue was subsequently sectioned in small fragments for biochemical determinations and RNA/miRNA isolation. Finally the faecal content was taken from each animal to do genomic DNA extraction. Then, amplification of 16S rRNA gene V1-V3 region and pyrosequencing of PCR amplicons were performed.

DNA extraction and 454/Roche pyrosequence analysis

DNA from faecal content was isolated using phenol:chloroform (protocol modified from (Sambrook J, 2001).

To compare how 16S rRNA gene sequence recovery was affected by storage and purification methods, total DNA from stool samples was PCR amplified using primers targeting regions flanking the variable regions 1 through 3 of the bacterial 16S rRNA gene (V1-3), gel purified, and analyzed using the 454/Roche GS FLX technology (Branford, CT, USA). The amplification of a 600-bp sequence in the variable region V1-V3 of the 16S rRNA gene was performed using barcoded primers. PCR was performed in a total volume of 15 µL for each sample containing the universal 27F and Bif16S-F forward primers (10 µmol/L) at a 9:1 ratio, respectively, and the barcoded universal reverse primer 534R (10 µ mol/L) in addition to dNTP mix (10 mmol/L), FastStart 10× buffer with 18 mmol/L of MgCl2, FastStart HiFi polymerase (5 U in 1 mL), and 2 µL of genomic DNA. The dNTP mix, FastStart 10× buffer with MgCl2, and FastStart HiFi polymerase were included in a FastStart High Fidelity PCR System, dNTP Pack (Roche Applied Science). The PCR conditions were as follows: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 5 min, and final step at 4°C. After PCR, amplicons were further purified using AMPure XP beads (Beckman-Coulter) to remove smaller fragments. DNA concentration and quality were measured using a Quant-iT™ PicoGreen® dsDNA Assay Kit. Finally, the PCR amplicons were combined in equimolar ratios to create a DNA pool (10^9 DNA molecules) that was used for clonal amplification (emPCR) and pyrosequencing according to the manufacturer's instructions. After the sequencing was completed, all reads were scored for quality, and any poor quality and short reads were removed.

The pipeline takes in bar coded sequence reads, separates them into individual communities by bar code, and utilizes a suite of external programs to make taxonomic assignments (Cole et al., 2007; Q. Wang, Garrity, Tiedje, & Cole, 2007) and estimate phylogenetic diversity.

Analysis of gene expression in mouse colonic samples by RT-qPCR

Total RNA from colonic samples was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA USA) and 2µg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK).

Real time quantitative PCR (qPCR) amplification and detection was performed on optical-grade 48-well plates in an Eco™ Real-Time PCR System (Illumina, CA, USA). Each reaction was composed of 5 µL of KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA), each amplification primer at a concentration of 10µM, 20 ng of cDNA from the RT reaction and PCR-grade water up to a final volume of 20 µL.

The thermal cycling program consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at annealing temperature (55-62ºC). Fluorescence was measured at the end of the annealing period of each cycle to monitor the progress of amplification, and dissociation curves were added to confirm the specificity of the amplification signal in each case. To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. For each sample, both the housekeeping and target genes were amplified in triplicate and the mean was used for further calculations. The mRNA relative quantitation was done using the $\Delta\Delta$Ct method. The specific primers used are indicated in table 5.

miRNA from colonic samples was isolated using QIAzol (Qiagen, Hilden, Germany). Tissue was homogenized in 1mL of QIAzol using a Precellys®24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Small RNA (<200 nt) fractions were isolated separately using miRNeasy mini Kit (Qiagen, Hilden, Germany) according to the Supplementary Protocol.

All miRNA samples were quantified with the Thermo Scientific NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA USA) and 500 ng of miRNA were reverse transcribed using the miScript II RT kit from Qiagen (Qiagen, Hilden, Germany).

Real time qPCR amplification and detection was performed on optical-grade 48-well plates in an Eco™ Real-Time PCR System (Illumina, CA, USA). Each reaction

was composed of 5 µL QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 1 µL miScript Universal Primer, 1 µL miScript Primer Assay, 2 ng of cDNA from the RT reaction and PCR-grade water up to a final volume of 10 µL.

The thermal cycling program consisted of an initial activation step of 15 min at 95 °C, followed by 40 cycles with 3-step clycing: 15 s at 94 °C for denaturation, the annealing step at 55ºC s for 30 s and 30 s at 70ºC for extension step. Fluorescence was measured at extension period of each cycle to monitor the progress of amplification, and dissociation curves were added to confirm the specificity of the amplification signal in each case. To normalize miRNA expression, the expression of the housekeeping gene, small nucleolar RNA, C/D box 95 (SNORD95) was measured. For each sample, both the housekeeping and target genes were amplified in triplicate and the mean was used for further calculations. The miRNA relative quantitation was done using the ΔΔCt method. The specific primers used are indicated in table 5.

IN VITRO STUDIES

The mouse carcinoma cells CMT-93 and bone marrow derived macrophages (BMDM) (obtained from Cell Culture Unit of the University of Granada, Granada, Spain) were grown in DMEM, supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine (2 mmol/l), penicillin (100 units/ml) and streptomycin (1 mg/ml), and maintained at 37ºC in a humidified, 5% CO_2 environment.

BMDM were generated as described previously (Celada, Gray, Rinderknecht, & Schreiber, 1984) with some modifications. Bone marrow was isolated from femurs of 6-week-old BALB/c mice (Janvier, St Berthevin Cedex, France). Mice were killed by cervical dislocation, the adherent tissue was removed and both femurs were dissected. The bone ends were cut off, and the marrow tissue was flushed by irrigation with DMEM. The marrow plugs were dispersed by passing them through a 25-gauge needle, and the cells were suspended by vigorous pipetting and washed. Cells were cultured in 150 mm Petri dishes with 40 ml of DMEM containing 20% FBS and 30% L-cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) (Comalada et al., 2006). Cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere. After 6 days of culture, a homogeneous population of adherent macrophages was obtained.

To evaluate the expression of different mRNAs, CMT-93 and BMDM cells were used. After reaching confluence, the cells were trypsinized and seeded onto 6-wells plates at a density of $5x10^5$ cells per well and grown until formation of a monolayer. They were pre-treated for 3 h with the different probiotics (*Lactobacillus fermentum* CECT5716, *Lactobacillus salivarius* CECT5713, *Escherichia coli* Nissle 1917, *Saccharomyces boulardii* CNCMI-745) suspended in DMEM at 10^8 colony forming units (CFU)/ml and 10^9 CFU/ml for *Saccharomyces boulardii* CNCMI-745. After this time, cells were stimulated with LPS, 10 μg/ml in CMT-93 and 10 ng/ml in BMDM, for 2 h. Untreated unstimulated cells and untreated cells were used as negative and positive controls. The supernatants were removed. Total cellular RNA was extracted from cells. The specific primers used are indicated in table 5.

EVALUATION OF THE PROBIOTIC VIABILITY ON THE INTESTINAL ANTI-INFLAMMATORY EFFECT EXERTED BY LACTOBACILLUS FERMENTUM

IN VIVO STUDIES

Trinitrobenzene sulphonic acid model of rat colitis

Female Wistar rats (180–200 g) were obtained from Janvier (St Berthevin Cedex, France). The rats were randomly assigned to four groups (n = 10); two of them (non-colitic and TNBS groups) received orally PBS solution (1 ml) and the other two (treated groups) were daily administered the probiotic orally, *Lactobacillus fermentum* CECT5716 (live and dead) at the concentration of $5x10^8$ CFU suspended in 1 ml of PBS solution, by means of an oesophageal catheter, for 3 weeks (Figure7). Two weeks after starting the experiment, the rats were fasted overnight, and those from the control and the treated groups were rendered colitic as previously described (Camuesco et al., 2005). Briefly, they were anaesthetized with isofluorane (Abbott Laboratories S.A., Madrid, Spain) and given 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) by means of a Teflon flexible cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically 0.25 ml of PBS instead of TNBS.

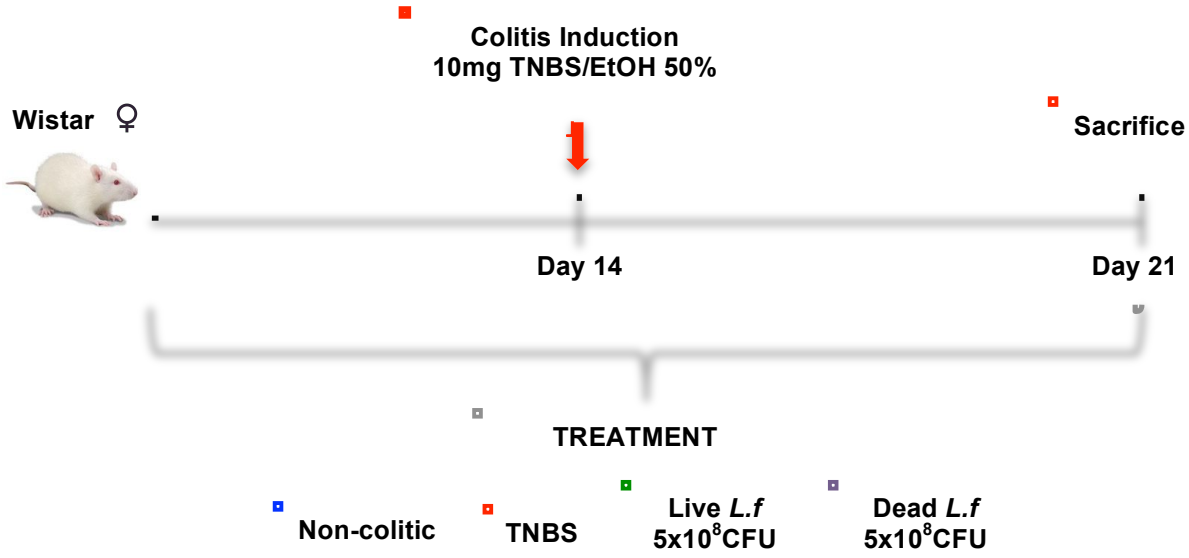


Figure 7. Experimental design in the TNBS model of rat colitis.

L.f (*Lactobacillus fermentum* CECT5716), *L.s* (*Lactobacillus salivariussalivarius* CECT5713), EcN (*Escherichia coli* Nissle 1917), *S.b* (*Saccharomyces boulardii* CNCMI-745)

Animal body weights, occurrence of diarrhea and water and food intake were recorded daily throughout all the experiments. Once the animals were sacrificed, the colon was removed aseptically and placed on an ice-cold plate, longitudinally opened and luminal contents were collected for the microbiological studies. Afterwards, the colonic segment was weighed and its length measured under a constant load (2 g). Each colon was scored for macroscopically visible damage on a 0-10 scale by two observers unaware of the treatment, according to the criteria described by Bell et al. (1995) (Bell et al., 1995) (Table 4).

Representative whole gut segments were obtained were subsequently sectioned in different longitudinal fragments to be used for biochemical determinations or for RNA isolation.

Table 4 . Criteria for assessment of macroscopic colonic damage in rat TNBS induced colitis. Described by Bell et al. (1995).

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

Evaluation of the intestinal inflammatory process

Biochemical determinations in colonic tissue.

- Mieloperoxidase activity.

Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al. (1984) (Krawisz et al., 1984). Colonic specimens where homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0) and MPO activity in supernatant was measured and calculated from the absorbance (at 460 nm) changes that resulted from decomposition of H_2O_2 in the presence of O-dianisidine; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 µmol hydrogen peroxide/min at 25ºC.

- Glutathione content.

Total glutathione (GSH) content was quantified with the recycling assay described by Anderson (1985) (Anderson, 1985) in which it is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase (Boehringer Mannheim, Barcelona, Spain). The rate of 2-nitro-5-thiobenzoic acid formation is monitored at 412 nm and the glutathione present was evaluated by comparison of that result with a standard curve, and the results were expressed as nmol/g wet tissue.

Analysis of gene expression in rat colonic samples by RT-qPCR.

Total RNA from colonic samples was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA USA) and 2µg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK).

Real time quantitative PCR (qPCR) amplification and detection was performed on optical-grade 48-well plates in an Eco^{TM} Real-Time PCR System (Illumina, CA, USA). Each reaction was composed of 5 µL of KAPA SYBR ® FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA), each amplification primer at a concentration of 10µM, 20 ng of cDNA from the RT reaction and PCR-grade water up to a final volume of 20 µL.

The thermal cycling program consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at annealing temperature (55-62ºC). Fluorescence was measured at the end of the annealing period of each cycle to monitor the progress of amplification, and dissociation curves were added to confirm the specificity of the amplification signal in each case. To normalize mRNA expression, the expression of the housekeeping gene, GAPDH was measured. For each sample, both the housekeeping and target genes were amplified in triplicate and the mean was used for further calculations. The mRNA relative quantitation was done using the $\Delta\Delta$Ct method. The specific primers used are indicated in Table 5.

IN VITRO STUDIES

The human colonic epithelial colorectal adenocarcinoma cell line Caco-2, the mouse macrophage RAW 264.7 cell line (obtained from Cell Culture Unit of the University of Granada, Granada, Spain) were grown in DMEM, supplemented with 10% FBS, L-glutamine (2 mmol/l), penicillin (100 units/ml) and streptomycin (1 mg/ml), and maintained at 37ºC in a humidified, 5% CO_2 environment.

To evaluate if the viability of *Lactobacillus fermentum* CECT5716 is essential to exert anti-inflammatory activity; both confluent cells in cell culture flasks were trypsinized and seeded onto 24-well plates at a density of 5×10^5 cells per well and grown until formation of a monolayer. Then they were pre-treated for 3 h with either live or dead bacteria suspended in DMEM at 10^8 CFU/ml. Cells were stimulated with different stimuli: LPS (100 ng/ml) (RAW 264.7) and IL-1β (1 ng/ml) (Caco-2) for 24 h to evaluate IL-8, IL-1β and nitrites levels, and for 30 min to evaluate p44/42 and p38 MAP kinase protein expression. Untreated unstimulated cells and untreated cells were used as negative and positive controls. Then, the supernatants were collected, centrifuged at 10000 g for 5 min and stored at − 80°C until cytokine and nitrites determinations were performed. The cytokine production was quantified by ELISA assay (R&D Systems, Abingdon, UK), whereas nitrite levels were measured using the Griess assay (Green et al., 1982), in which Griess reagents (0.1 % N-(1-naphthy) ethylenediamine solution and 1% sulphanilamide in 5% (v/v) phosphoric acid solution) convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance at 550 nm due to this azo chromophore accurately determines nitrite concentration (Granger, Anstey, Miller, & Weinberg, 1999). The p44/42 MAP kinase protein expression in Caco-2 cells was performed by immunoblotting (Hisamatsu & King, 2008). Equal amounts of protein from samples (50 µg) were separated on 10 % SDS-PAGE. Phosphorilated p44/42 MAP kinase antibody (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain) was used at a dilution of 1/2500. p44/42 MAP kinase antibody was used as loading control. Peroxidase-conjugated anti-mouse IgG were used as secondary antibodies. Then, ECL (Perkin ElmerTM, Life Sciences, Boston, USA) detection was performed. Cell viability was examined by the MTT-test following the manufacturer's instructions (Mosmann, 1983), and it was not affected by the treatments.

STATISTICS

All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post-hoc least significance tests. Non-parametric data (score) are expressed as the median (range) and were analyzed using the Mann–Whitney U-test. Differences between proportions were analyzed with the chi-square test. All statistical analyses were carried out with the GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA, USA), with statistical significance set at $P<0.05$.

REAGENTS

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

Table 5. Primer sequences used in real-time qPCR assays.

Gene	Sequence (5'-3')	Annealing temperature (ºC)
TNF-α	F:AACTAGTGGTGCCAGCCGAT R:CTTCACAGAGCAATGACTCC	56
IL-1β	F:TGATGAGAATGACCTGTTCT R:CTTCTTCAAAGATGAAGGAA	55
IL-6	F: CCGGAGAGGAGACTTCACAG R: GGAAATTGGGGTAGGAAGGA	62
IL-12	F:CCTGGGTGAGCCGACAGAAGC R:CCACTCCTGGAACCTAAGCAC	60
TGF-β	F:GCTAATGGTGGACCGCAACAAC R:CACTGCTTCCCGAATGTCTGAC	60
MCP-1	F:AGCCAACTCTCACTGAAG R:TCTCCAGCCTACTCATTG	60
ICAM-1	F:GAGGAGGTGAATGTATAAGTTATG R:GGATGTGGAGGAGCAGAG	60
iNOS	F:GGCAGAATGAGAAGCTGAGG R:GAAGGCGTAGCTGAACAAGG	55
MMP-2	F:TGCCGGCACCACTGAGGACTAC R:GGGCTGCCACGAGGAACA	56
MMP-9	F:TGGGGGGCAACTGGGC R:GGAATGATCTAAGCCCAG	60
MUC-2	F:GATAGGTGGCAGACAGGAGA R:GCTGACGAGTGGTTGGTGAATG	60
MUC-3	F:CGTGGTCAACTGCGAGAATGG R:CGGCTCTATCTCTACGCTCTCC	62
ZO-1	F:GGGGCCTACACTGATCAAGA R:TGGAGATGAGGCTTCTGCTT	56
OCLN	F:ACGGACCCTGACCACTATGA R:TCAGCAGCAGCCATGTACTC	56
GAPDH	F:CATTGACCTCAACTACATGG R:GTGAGCTTCCCGTTCAGC	55
miR-143	UGAGAUGAAGCACUGUAGCUC	55
miR-150	UCUCCCAACCCUUGUACCAGUG	55
miR-155	UUAAUGCUAAUUGUGAUAGGGGU	55
miR-223	UGUCAGUUUGUCAAAUACCCCA	55
miR-375	UUUGUUCGUUCGGCUCGCGUGA	55

Results

RESULTS

PREVENTIVE EFFECT OF PROBIOTICS IN ACUTE MODELS OF RODENT COLITIS: IMPACT ON miRNA EXPRESSION AND INTESTINAL MICROBIOTA COMPOSITION

1. Evaluation of the intestinal antiinflammatory effects of the probiotics in the DSS-model of mouse colitis.

The intestinal anti-inflammatory effect of different probiotics: *Lactobacillus fermentum* CECT5716, *Lactobacillus salivarius* CECT5713, *Escherichia coli* Nissle 1917 and *Saccharomyces boulardii* CNCMI-745 were studied in the DSS model of mouse colitis, a well established model with some resemblance to human UC (Okayasu et al., 1990). A preventative dosing protocol was followed in all cases; firstly, because this has been the protocol used in previous studies to report the intestinal anti-inflammatory effect of these probiotics (Arribas et al., 2009; Garrido-Mesa et al., 2015; Peran et al., 2006; Peran et al., 2005), and secondly, because preliminary experiments performed by our group have revealed that the administration of probiotics and/or prebiotics once the colonic damage has been induced has a deleterious effect (unpublished observations). According to this, the different probiotics were administered daily for all the experimental period, at the dose of $5x10^9$ CFU/mouse *Sacharomyces boulardii* and all others at $5x10^8$ CFU/mouse, starting 14 days before colitis induction, which was performed by incorporating DSS in the drinking water for 6 days, as well as thereafter until the sacrifice of the animals that took place 6 days after the removal of the DSS. It is important to note that the administration of the different probiotics for 2 weeks before colitis induction did not result in any symptom of diarrhoea or affect weight evolution (data not shown).

The administration of 3% (w/v) DSS dissolved in the drinking water for 6 days to mice resulted in a progressive increase in DAI values, due to the body weight loss and the excretion of diarrheic/bleeding feces (Figure 8). However, the oral treatment with all the probiotics studied attenuated the impact of the DSS damage as well as improved the recovery of colitic mice, as evidenced by the reduced weight loss and the lower incidence in the presence of diarrhoeic/bloody faeces, thus obtaining lower DAI values during the time-course of the experiment in the treated groups in comparison to untreated colitic mice (Figure 8). The macroscopic evaluation of the colonic segments

confirmed the beneficial observed in probiotic-treated colitic mice, since they showed a significant reduction in colonic weight/length ratio when compared with colitic control mice (Figure 8); of note, this is a parameter that has been suggested to be directly correlated with the severity of the colonic damage in the DSS-induced colitis (Arafa, Hemeida, El-Bahrawy, & Hamada, 2009).

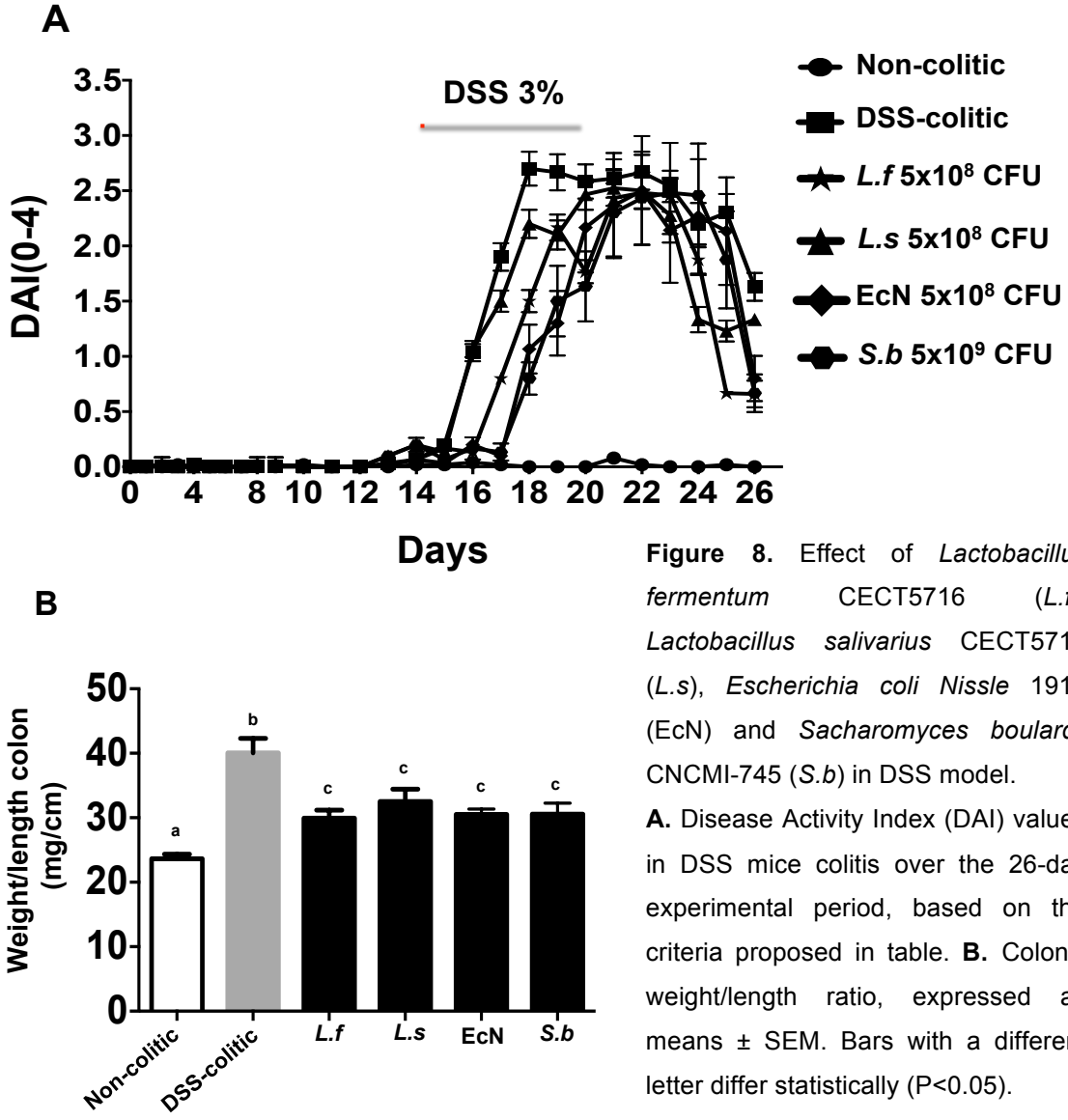


Figure 8. Effect of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*) in DSS model.
A. Disease Activity Index (DAI) values in DSS mice colitis over the 26-day experimental period, based on the criteria proposed in table. **B.** Colonic weight/length ratio, expressed as means ± SEM. Bars with a different letter differ statistically (P<0.05).

Furthermore, the biochemical analysis of the colonic segments corroborated the intestinal anti-inflammatory effects of both lactobacilli, revealing the positive impact on the altered colonic immune response. Thus, the probiotic treatments significantly reduced the upregulated expression of the proinflammatory cytokine IL-1β observed in control colitic mice in comparison with animals from non-colitic group (Figure 9.A).

When IL-12 was analysed, only *L. fermentum* CECT5716 and *E. coli* Nissle 1917 significantly downregulated the increased expression observed in control colitic mice (Figure 9.A). However, no significant modification was obtained on colonic TNF-α expression among colitic groups. The inflammatory colonic process induced by DSS was associated with a reduced expression of TGF-β, a mediator that displays both anti-inflammatory and pro-inflammatory processes, depending on the local cytokine milieu (Fantini et al., 2007); the treatment with any of the probiotic assayed resulted in the significant increase of this cytokine expression to values similar to non-colitic mice (Figure 9.A). The analysis of the chemokine MCP-1 showed that none of the probiotics were able to significantly modify the increased expressions observed in control colitic mice in comparison with the non-colitic group; however, only *E. coli* Nissle 1917 significantly reduced the expression of the adhesion molecule ICAM-1 (Figure 9.B). The colonic expressions of metalloproteinases MMP-2 and MMP-9 were also increased in those mice from the colitic control group. Excepting *E. coli* Nissle 1917 in MMP-9, all the probiotic treatments significantly reduced the expression of these enzymes, without showing statistical differences with the non-colitic group (Figure 9.B). Finally, the expression of the inducible enzyme iNOS was significantly upregulated in DSS-induced colitic mice, and only those animals receiving *L. fermentum* showed a significant reduction in comparison with the control colitic mice (Figure 9.B). As it has been previously described, the DSS-induced colonic inflammation was associated with a reduced expression of different proteins related with epithelial integrity, like the mucins MUC-3 and MUC-2, occludin and ZO-1. The groups of colitic mice that received the lactobacilli, *E. coli* Nissle 1917 or *S. boulardii* showed a significant amelioration in the modification of all these markers, except for *L. salivarius* and *S. boulardii*, which were not able to significantly increase ZO-1 expression, although a trend was obtained and no statistical differences were obtained with the non-colitic group (Figure 9.B).

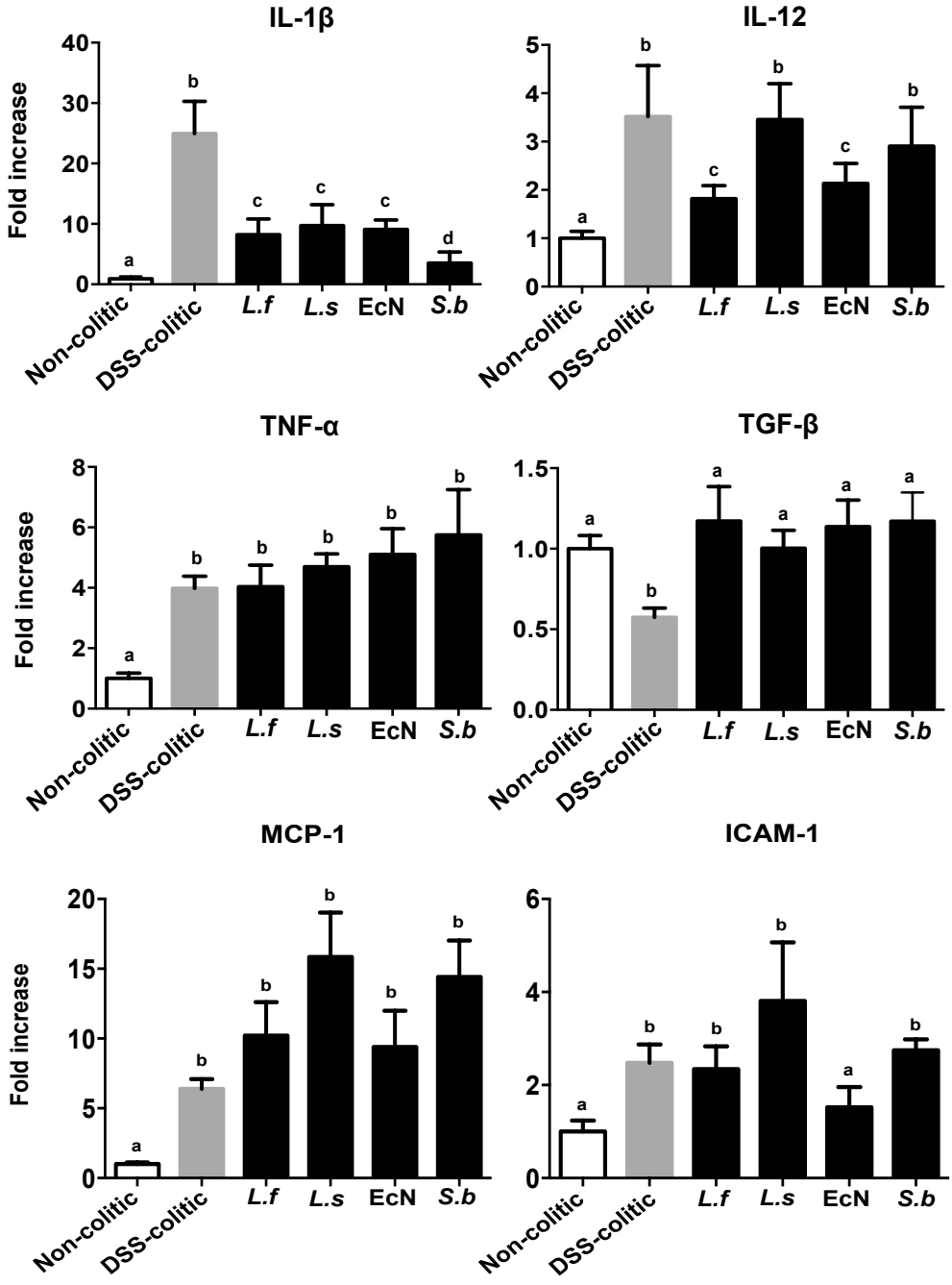


Figure 9.A. Biochemical evaluation of the effects of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*); mRNA expression of pro-inflammatory cytokines IL-1β, IL-12, TNF-α and TGF-β; chemokine MCP-1; and ICAM-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM. Bars with a different letter differ statistically ($P<0.05$).

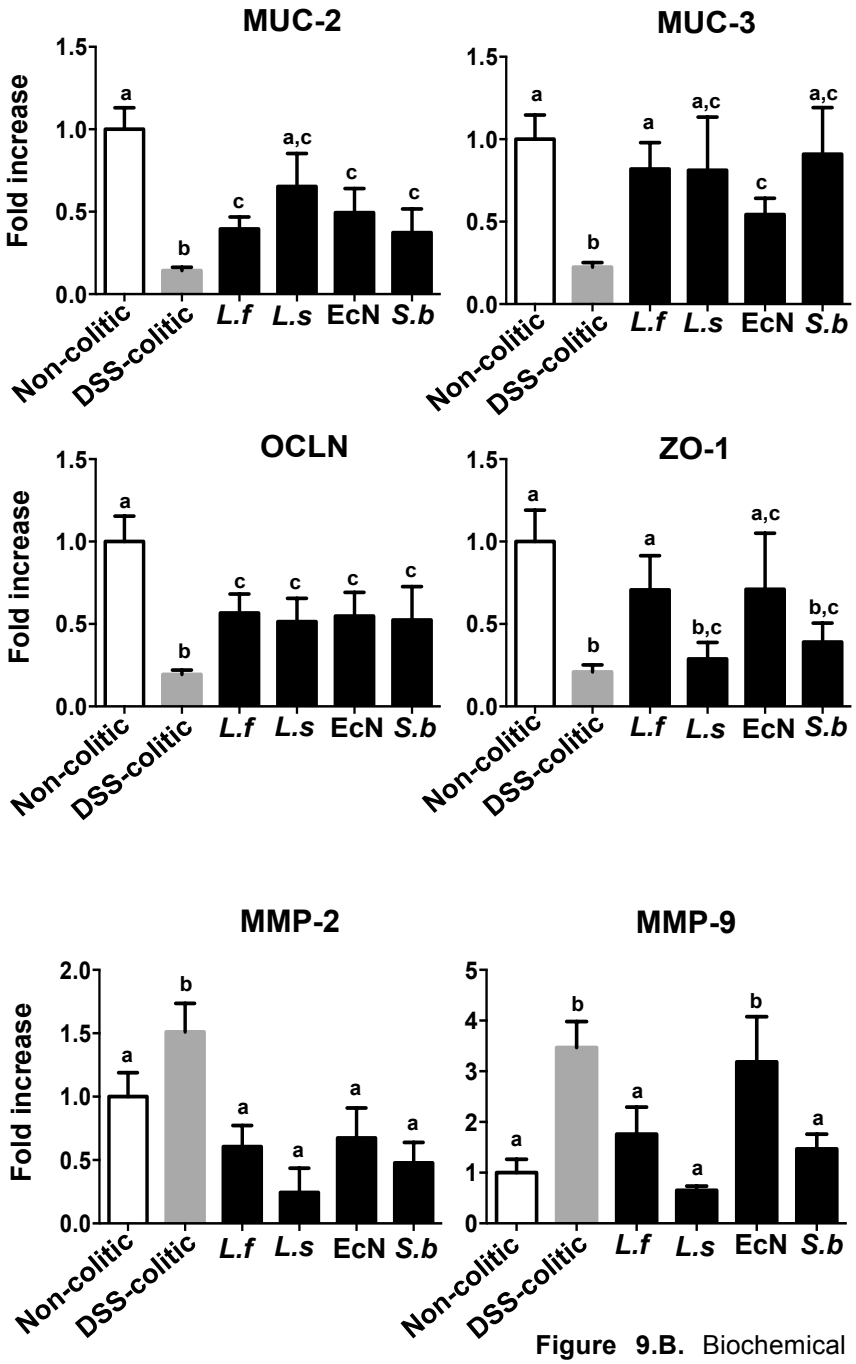


Figure 9.B. Biochemical evaluation of the effects of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*); mRNA expression of epithelial protein expression MUC-2, MUC-3, OCLN, ZO-1; and enzymes MMP-2, MMP-9 and iNOS was quantified by real-time PCR, and fold increases are expressed as means ± SEM. Bars with a different letter differ statistically (P<0.05).

The results obtained in this experimental model of colitis induced with DSS confirmed that the intestinal inflammatory process was associated with noticeable molecular changes in gene and protein expression. Also, these results support that the restoration of the altered immune response is involved the preventive beneficial effects exerted by the probiotics assayed in experimental colitis. Then, we explored if miRNAs, which have been reported to play an important role in many biological processes, such as signal transduction, cellular proliferation, differentiation, apoptosis and immune response (O'Connell et al., 2012), could be also associated with the response obtained after probiotic treatment. With this aim, the expressions of selected miRNAs were also evaluated by qPCR. In fact, miRNA expressions of miR-143 and miR-375 were significantly decreased in colitic mice in comparison with non-colitic group, whereas miR-150, miR-155 and miR-223 showed increased expression after colonic insult with DSS. The treatment with the probiotics resulted in the amelioration of the altered expression in some of the miRNAs evaluated. Thus, all the probiotics were able to significantly reduce the upregulated expressions of miR-223 and miR-155, but when miR-150 expression was considered, it was restored in colitic mice treated with *L. fermentum* or *E.coli* Nissle 1917. Likewise, only *L. salivarius* was able to significantly increase the expression of miR-375 in comparison with control colitic mice, while *L. fermentum* and *E.coli* Nissle 1917 showed a similar effect when miR-143 was evaluated (Figure 10).

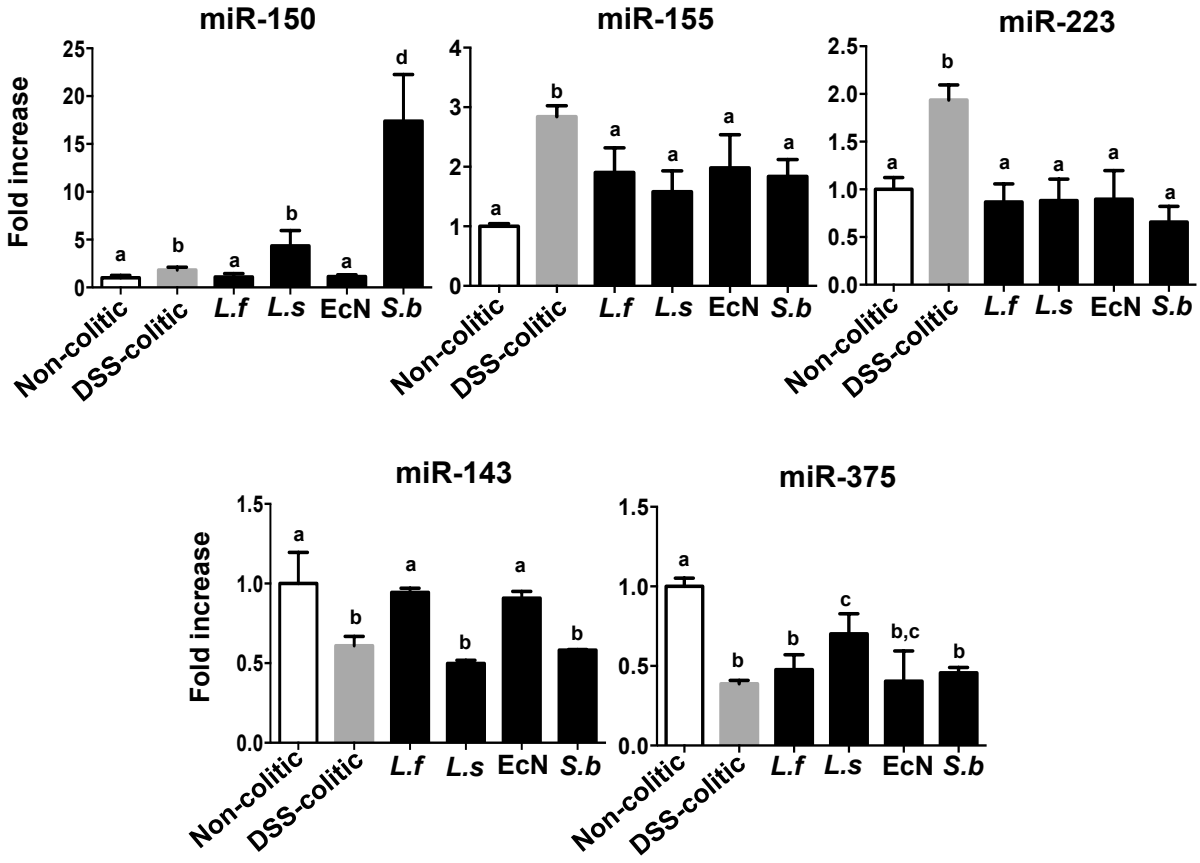


Figure 10. Biochemical evaluation of the effects of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*)*;* miRNA expression of miR-143, miR-150, miR-155, miR-223 and miR-375 was quantified by real-time PCR, and fold increases are expressed as means ± SEM. Bars with a different letter differ statistically (P<0.05).

Subsequently, and in order to study the modifications in the intestine microbiota composition in colitic mice after DSS insult, 16S ribosomal DNA sequencing and bioinformatics alignment comparison against ribosomal data project (RDP) database were performed. The compositions of bacterial communities were evaluated by calculating three major ecological parameters, including Chao richness (an estimate of a total community), Pielou evenness (to show how evenly the individuals in the community are distribuited over different operational taxonomic units) and Shannon diversity (the combined parameter of richness and evenness). Microbial richness, evenness, and diversity were all found to be drastically decreased in the DSS-colitic group when compared with Non-colitic. When the microbiota composition in the intestinal contents was evaluated in the probiotic-treated colitic groups, the results

showed that *L. fermentum* and *E.coli* Nissle 1917 were able to significantly increase all these ecological parameters studied. However, *S. boulardii* only showed statistical significance with Chao richness in comparison with control colitic group (Figure 11).

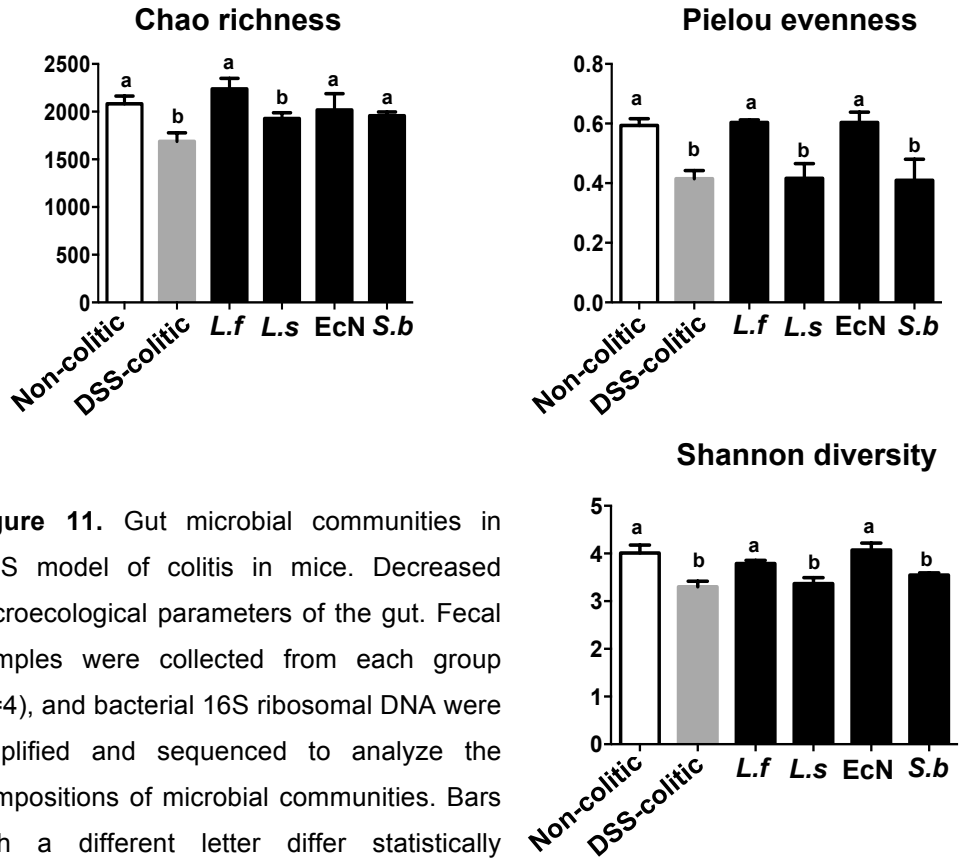
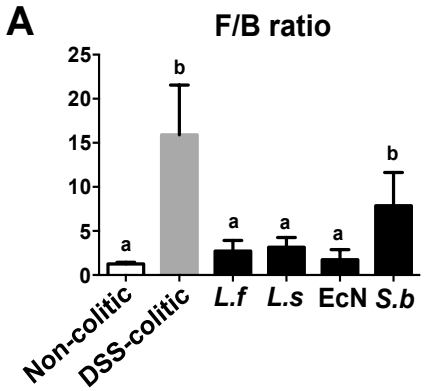


Figure 11. Gut microbial communities in DSS model of colitis in mice. Decreased microecological parameters of the gut. Fecal samples were collected from each group (n=4), and bacterial 16S ribosomal DNA were amplified and sequenced to analyze the compositions of microbial communities. Bars with a different letter differ statistically ($P<0.05$).

Finally, the changes in the ratio of the microbiome communities *Firmicutes* (F) and *Bacteriodetes* (B), known as the F/B ratio, which can be potentially used as a biomarker for pathological conditions as obesity, inflammation and diarrhea conditions (Mariat D et al, 2009; Sanz Y, Moya-perez A, 2014, Youmans BP et al, 2015), were also analysed. Additionally, the mean abundances of the phyla *Actinobacteria* or *Proteobacteria*, which are present in smaller proportions, were also evaluated in the colonic contents from the different experimental groups. In the present study, the F/B ratio in DSS-colitic control mice was significantly increased (≈ 10-fold) when compared with non-colitic mice (Figure 12.A). It is interesting to note that when this parameter was evaluated in treated colitic gropus, only S*. boulardii* were not able to decrease this ratio. Also, probiotic treatments, except *E.coli* treatment, trend to restore the abundance of *Actinobacteria* and *Proteobacteria*, which was reduced in the DSS-

control group. Similarly, the abundance of *Bacteriodetes* and *Firmicutes* phylum were restored by all probiotics treatment less *S.boulardii*. Finally, when the phylum *Proteobacteria* was considered, only the yeast was able to restore this abundance (Figure 12.B).

A



B

Phylum	Mean abundance (%)					
	Non-colitic	DSS-colitic	*L.f*	*L.s*	EcN	*S.b*
Actinobacteria	4.77 [a]	0.37 [b]	1.31 [c]	1.23 [c]	0.64 [d]	3.25 [a]
Bacteriodetes	38.90 [a]	7.70 [b]	30.44 [a]	25.60 [a]	42.74 [a]	14.59 [c]
Firmicutes	47.46 [a]	81.11 [b]	61.15 [c]	57.92 [c]	42.27 [a]	76.60 [b]
Proteobacteria	1.20 [a]	1.26 [a]	0.64 [c]	0.50 [c]	0.91 [a,c]	1.17 [a]

Figure 12. Comparison of microbiota between Non-colitic group, DSS-colitic, *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*). **A.** The *Firmicutes/Bacteriodetes* ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis. **B.** Phylum breakdown of the most abundant bacterial communities in the different groups. Bars with a different letter differ statistically ($P<0.05$).

2. Evaluation of the intestinal antiinflammatory effects of the probiotics in the DNBS-model of mouse colitis.

Once the intestinal anti-inflammatory effects of these probiotics were evidenced in the DSS model of mice colitis, and the involvement of their immunomodulatory properties characterized, as well as the impact on miRNAs expression and microbiota

composition, we next evaluated the preventative properties of the same probiotics in another experiment model of colitis. This was induced by the intrarrectal administration of DNBS to mice. This model is characterized by an intense acute inflammatory response with some resemblance to human CD (Morampudi et al., 2014). The dosing protocol used was similar to that described in the DSS model, and probiotics were given orally an daily at the dose of $5x10^8$ CFU/mouse, except *Sacharomyces boulardii* that was given at $5x10^9$ CFU/mouse, for 20 days before colitis induction, and thereafter until the sacrifice of the animals that took place 4 days after DNBS instillation. As previously commented, the administration of the different probiotics for approximately 3 weeks before colitis induction did not result in any symptom of diarrhoea or affect weight evolution.

DNBS administration resulted in the onset of an intestinal inflammatory process that was characterized by a progressive reduction in body weight with time, associated with a reduced food intake, and the presence of diarrheic faeces in the majority of the colitic mice. In contrast with the results obtained when probiotics were evaluated in DSS colitis, in the DNBS model, the probiotic did not show any beneficial effect on weight evolution during the 4 days after colonic insult (Figure 13). In fact, once the mice were sacrificed for evaluation of the colonic segments, the inflammatory process was macroscopically characterized by a significant increase in the colonic weigth/length ratio in comparison with non-colitic mice, but no significant modifications were obtained in this parameter in the groups of mice that received the probiotic treatment when compared with the corresponding colitic control (Figure 13).

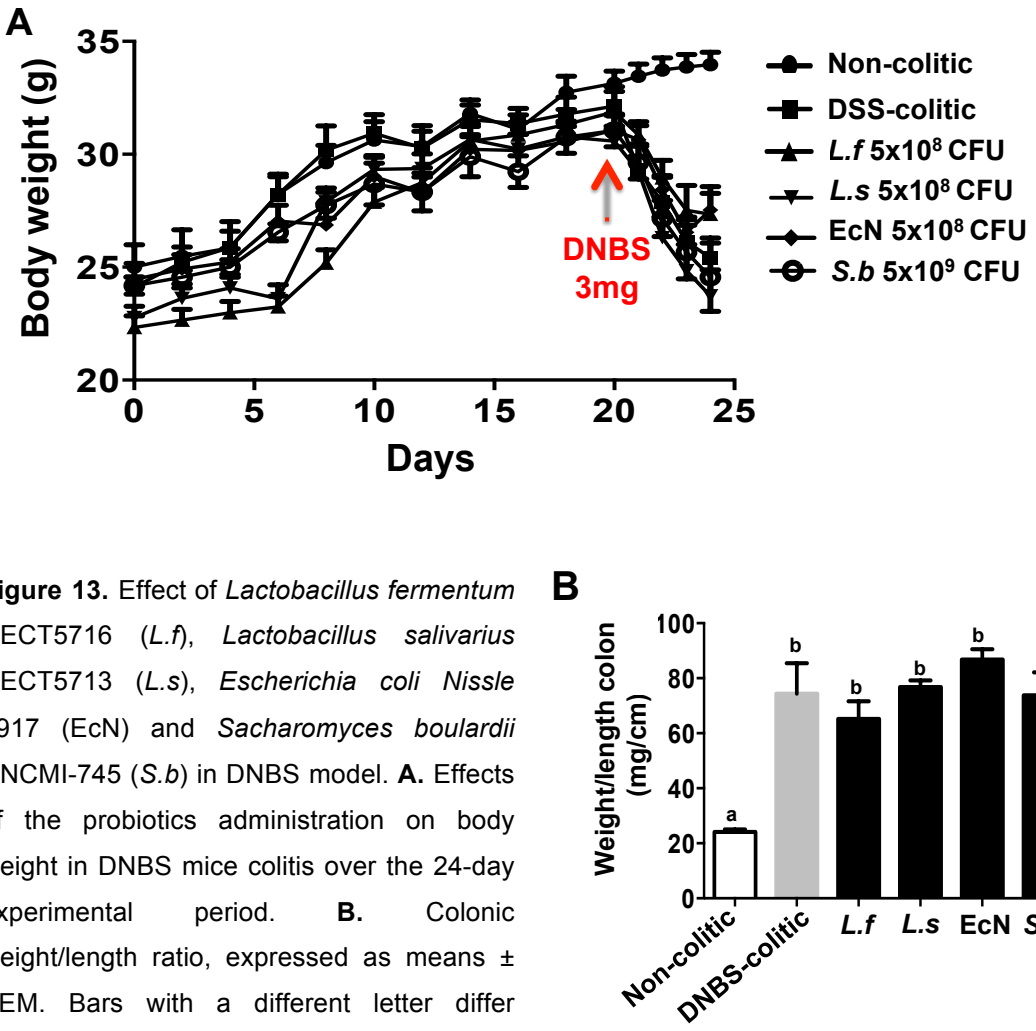


Figure 13. Effect of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*) in DNBS model. **A.** Effects of the probiotics administration on body weight in DNBS mice colitis over the 24-day experimental period. **B.** Colonic weight/length ratio, expressed as means ± SEM. Bars with a different letter differ statistically (P<0.05).

The colonic inflammatory status in the DNBS model of colitis was also characterized by the existence of an altered immune response, as shown by an up-regulated expression of the proinflammatory cytokines IL-1β and TNF-α. Also, and in comparison with non-colitic animals, the expressions of the inducible enzymes iNOS and MMP-2 were upregulated, together with and a decreased expression of the MUC-3 and occludin, both proteins involved in the maintaining of intestinal epithelial integrity (Figure 14). Although no beneficial effect was observed when colonic inflammation was evaluated macroscopically, the biochemical analysis of the intestinal specimens revealed the positive impact of probiotic treatment on the immune response. Thus, *L. fermentum* and *E. coli* Nissle 1917 were able to significantly reduce the expression of

both IL-1β and TNF-α in comparison with untreated colitic mice, whereas those animals receiving *L. salivarius* and *S. boulardii* only showed a significant down-regulation of IL-1β expression (Figure 14). All the probiotics significantly reduced the colonic expression of MMP-2 in colitic mice, except *S. boulardii*, although a trend was observed in this parameter. However, only in those mice treated with *L. fermentum,* a reduced expression of colonic iNOS was observed in comparison with untreated control mice. Finally, all the probiotics showed beneficial effects when the proteins involved in epithelial integrity were evaluated, since they significantly upregulated the expressions of the mucin MUC-3 and occludin, obtaining in most of the cases a similar expression to that observed in non-colitic mice (Figure 14).

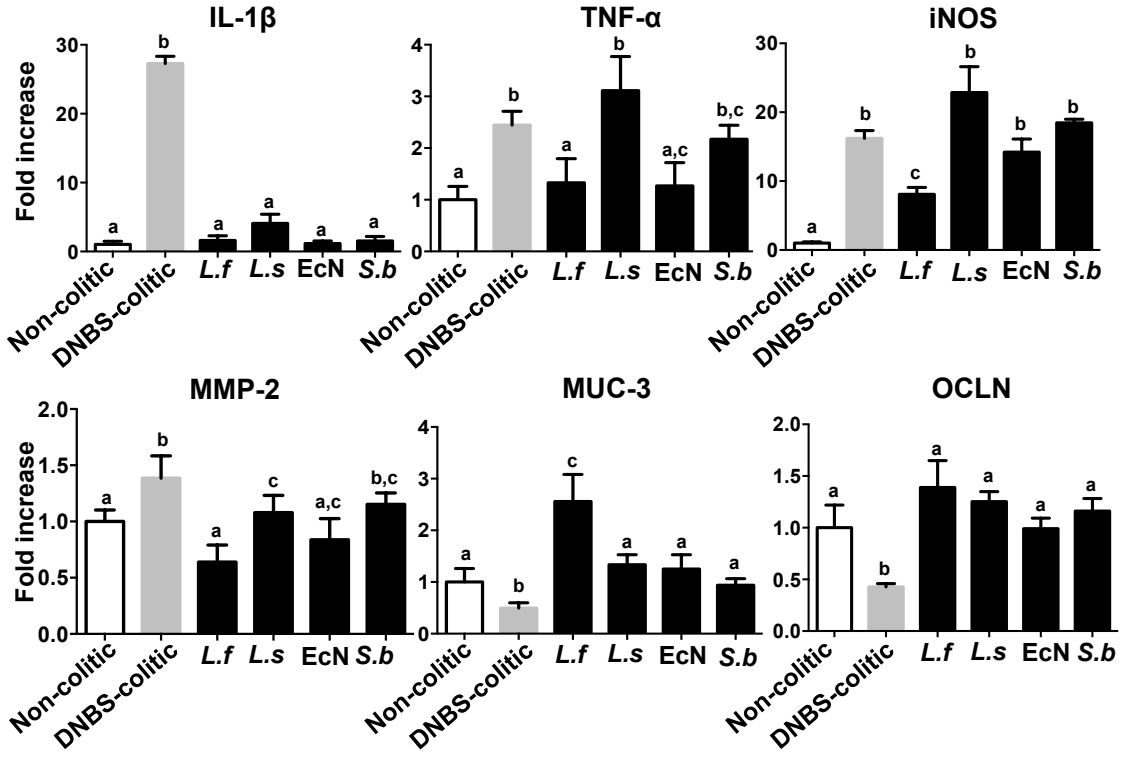


Figure 14. Biochemical evaluation of the effects of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*) after 24 days of treatment; mRNA expression of IL-1β, TNF-α, iNOS, MMP-2, MUC-3 and OCLN was quantified by real-time PCR, and fold increases are expressed as means ± SEM; Bars with a different letter differ statistically ($P<0.05$).

The inflammatory process induced by DNBS in mice was also associated with significant modifications in the expression of the different miRNA evaluated. Thus, the expressions of miR-155 and miR-223 were increased in DNBS-colitic mice when compared with healthy mice, whereas miR-143, miR-150 and miR-375 expressions were reduced as a consequence of the colonic insult (Figure 15). Besides, the treatments promoted the recovery in these markers, although not all the probiotics showed the same profile when considering the different miRNAs evaluated; with the exception of miR-150, all probiotic treatments were able to restore this expression. Thus, and when compared with untreated colitic mice, *L. fermentum* administration significantly increased the expression of miR-143 and reduced those of miR-155 and miR-223, without showing statistical differences when miR-375 was analysed. The treatment of colitic mice with *L. salivarius* resulted in the increased expression of miR-375, together with a reduction in both miR-155 and miR-223, but no significant modification was observed on miR-143 expression. Colitic mice administered with *E. coli* Nissle 1917 showed increased expression of miR-143 while reducted miR-223 expression. Finally, the administration of *S. boulardii* only showed a significant effect when miR-223 expression was evaluated in colitic mice, obtaining similar values to those obtained with non-colitic mice (Figure 15).

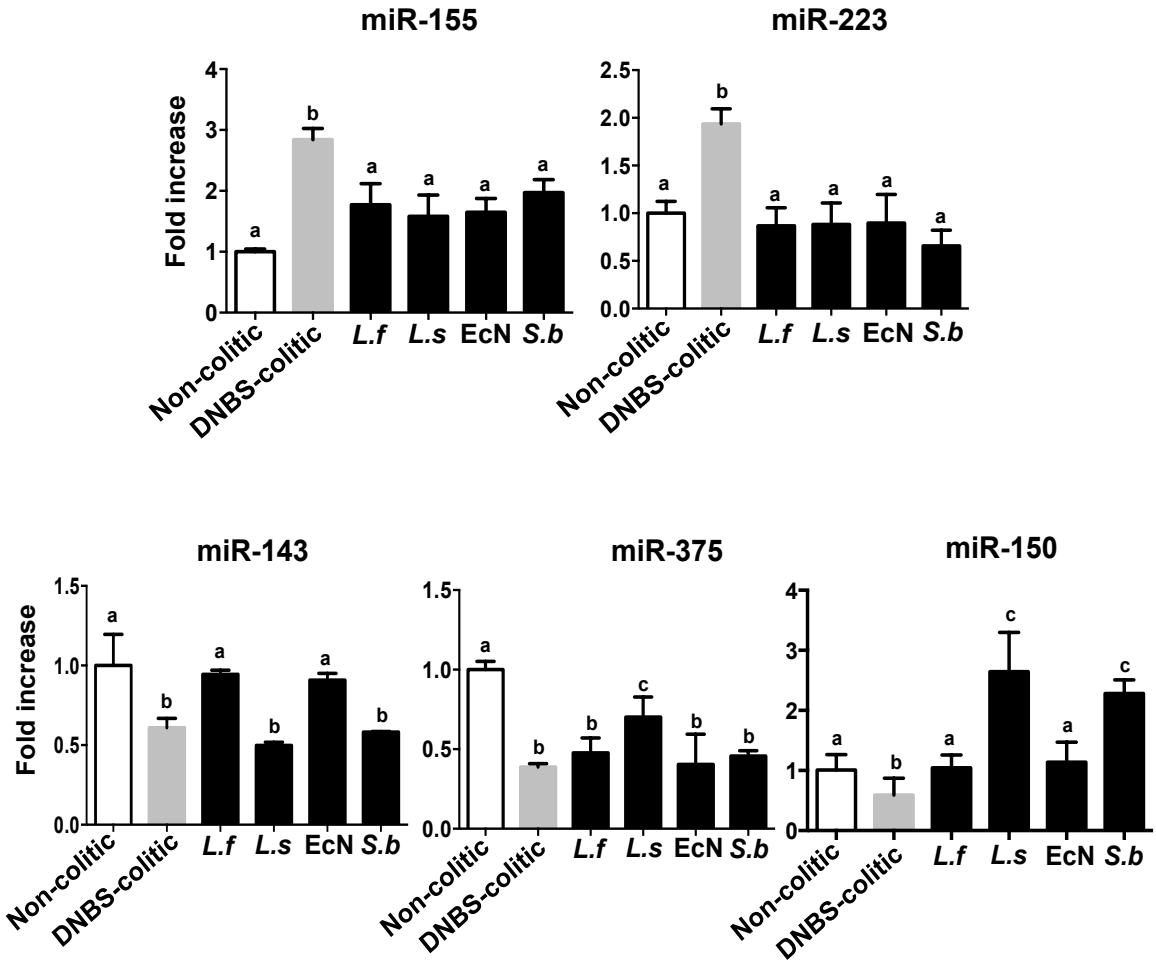


Figure 15. Biochemical evaluation of the effects of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*) after 24 days of treatment; miRNA expression of miR-150, miR-155, miR-223, miR-143 and miR-375 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; Bars with a different letter differ statistically ($P<0.05$).

When the microbiota composition was evaluated in this experimental model of colitis by 16S rDNA sequencing, the statistical analysis of the data revealed that DNBS-colitic mice only showed a lower diversity value when compared with non-colitic group, as evidenced by a reduced Shannon index. This lower diversity was significantly increased by probiotic treatment to colitic mice with lactobacilli or with *E. coli* Nissle 1917, but not with *S. boulardii* (Figure 16).

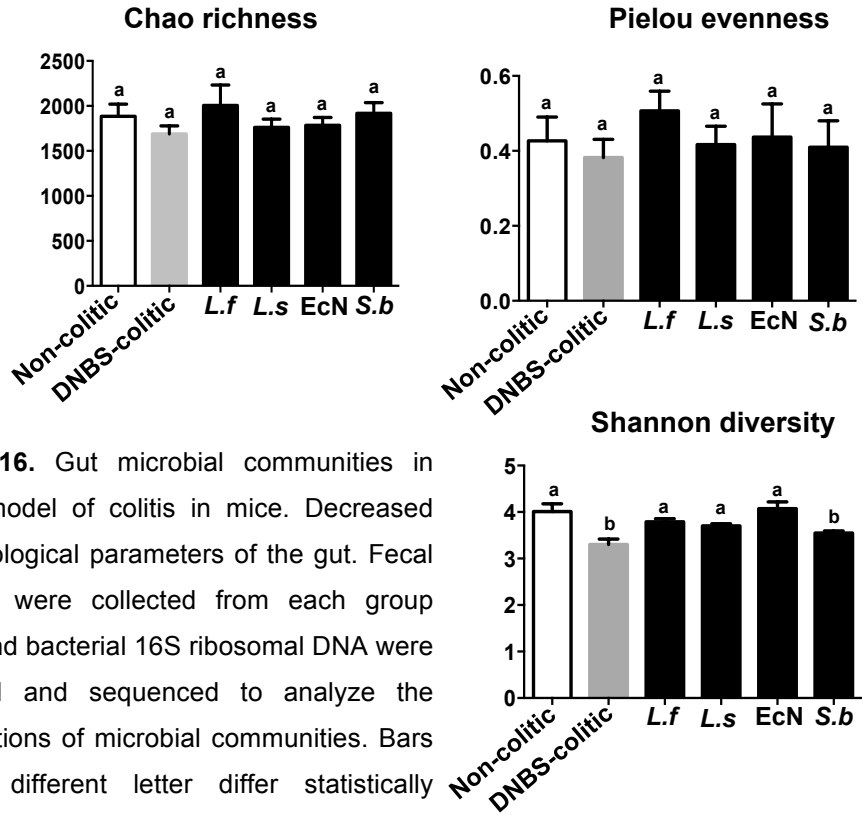
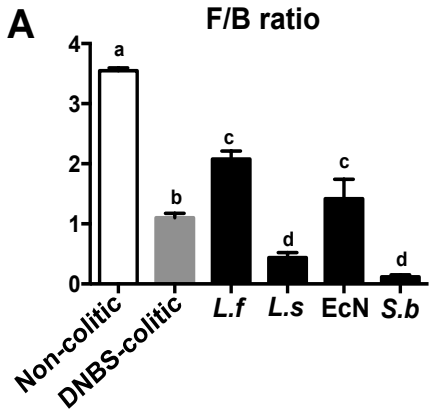


Figure 16. Gut microbial communities in DNBS model of colitis in mice. Decreased microecological parameters of the gut. Fecal samples were collected from each group (n=4), and bacterial 16S ribosomal DNA were amplified and sequenced to analyze the compositions of microbial communities. Bars with a different letter differ statistically (P<0.05).

Similarly to data permorfed in DSS model, the abundance of the principal phyla was evaluated, and the F/B ratio was calculated. The results revealed that the F/B ratio value in the DNBS group showed significant differences compared with non-colitic group (Figure 17.A). The reduced F/B ratio in DNBS mice was mainly related to a significant reduction of abundance in *Firmicutes*. The *L.fermentum* and *E.coli* treatment were able to increase this ratio (Figure 17.A). Additionally, the abundance of *Actinobacterias* and *Proteobacterias* was also calculated. The colitic group showed a significant modification compared with non-colitic mice, and the probiotic treatments did not modify the abundance of any phylum (Figure 17.B).



B

Phylum	Mean abundance (%)					
	Non-colitic	DNBS-colitic	*L.f*	*L.s*	EcN	*S.b*
Actinobacteria	31.74 [a]	42.24 [b]	44.23 [b]	40.56 [b]	40.74 [b]	62.77 [c]
Bacteriodetes	12.48 [a]	19.23 [a]	12.22 [a]	29.67 [b]	18.84 [a]	14.77 [a]
Firmicutes	44.38 [a]	21.11 [b]	29.65 [b]	13.02 [c]	26.78 [b]	1.81 [d]
Proteobacteria	0.60 [a]	0.17 [b]	0.17 [b]	1.08 [c]	0.11 [b]	15.75 [d]

Figure 17. Comparison of microbiota between Non-colitic group, DNBS-colitic, *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*). **A.** The Firmicutes/Bacteriodetes ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis. **B.** Phylum breakdown of the most abundant bacterial communities in the different groups. Bars with a different letter differ statistically ($P<0.05$).

3. In vitro effects of probiotics in CMT-93 and BMDM cells.

In order to better characterize the immunomodulatory properties of the probiotics used in the present study (*Lactobacillus fermentum* CECT5716, *Lactobacillus salivarius* CECT5713, *Escherichia coli* Nissle 1917 and *Saccharomyces boulardii* CNCMI-745), and to examine their role in the intestinal anti-inflammatory effect showed in the in vivo experiments, the probiotics were assayed in vitro in two cell cultures that represent different cell types involved in the intestinal immune response: the mouse carcinoma cell line CMT-93 was used as a model of intestinal

epithelial cells, and the primary culture BMDM as a model of macrophages. These studies were performed by incubating the probiotics in two different experimental conditions: basal and when the cells were subsequently stimulated with LPS, thus inducing several immediate pro-inflammatory responses (Morrison & Ulevitch, 1978). The cell responses in the different experimental conditions were evaluated by determining the expression of different markers that are related with the intestinal inflammatory response that occurs in vivo, that is, cytokines, chemokines and mucins.

When the CMT-93 cells were incubated in the presence of the different probiotics for three hours, only *E. coli* Nissle 1917 was able to significantly increase de expression of the proinflammatory cytokines evaluated: TNF-α and IL-6. In these basal conditions, both *L. fermentum* and *E. coli* Nissle 1917 upregulated the expression of the mucin MUC-3. The incubation of these epithelial cells with LPS resulted mimicked an inflammatory cell environment as evidenced by the increased expression of the proinflammatory cytokines TNF-α and IL-6, as well as a significant reduction in the expression of the mucin MUC-3. The pretreatment of these cells with the different probiotics, resulted in amelioration of the expression of some of these markers, but revealing differences among them. Thus, when considering the lactobacilli, only *L. fermentum* was able to inhibit the expression of one of the pro-inflammatory cytokines evaluated, IL-6. Of note, both lactobacilli upregulated the expression of MUC-3, being this expression even higher than that obtained in untreated cells. The pre-treatment of the epithelial cells with *E. coli* Nissle 1917 before LPS stimulation resulted in a significant decreased expression of both proinflammatory cytokines assayed, TNF-α and IL-6; and was also able to significantly increase the reduced expression of MUC-3 induced by LPS in these epithelial cells. Finally, *S. boulardii* was also able to affect the cell response after LPS stimulation, since this yeast was able to significantly decrease the expression of IL-6, as well as to upregulate that of the mucin MUC-3 (Figure 18).

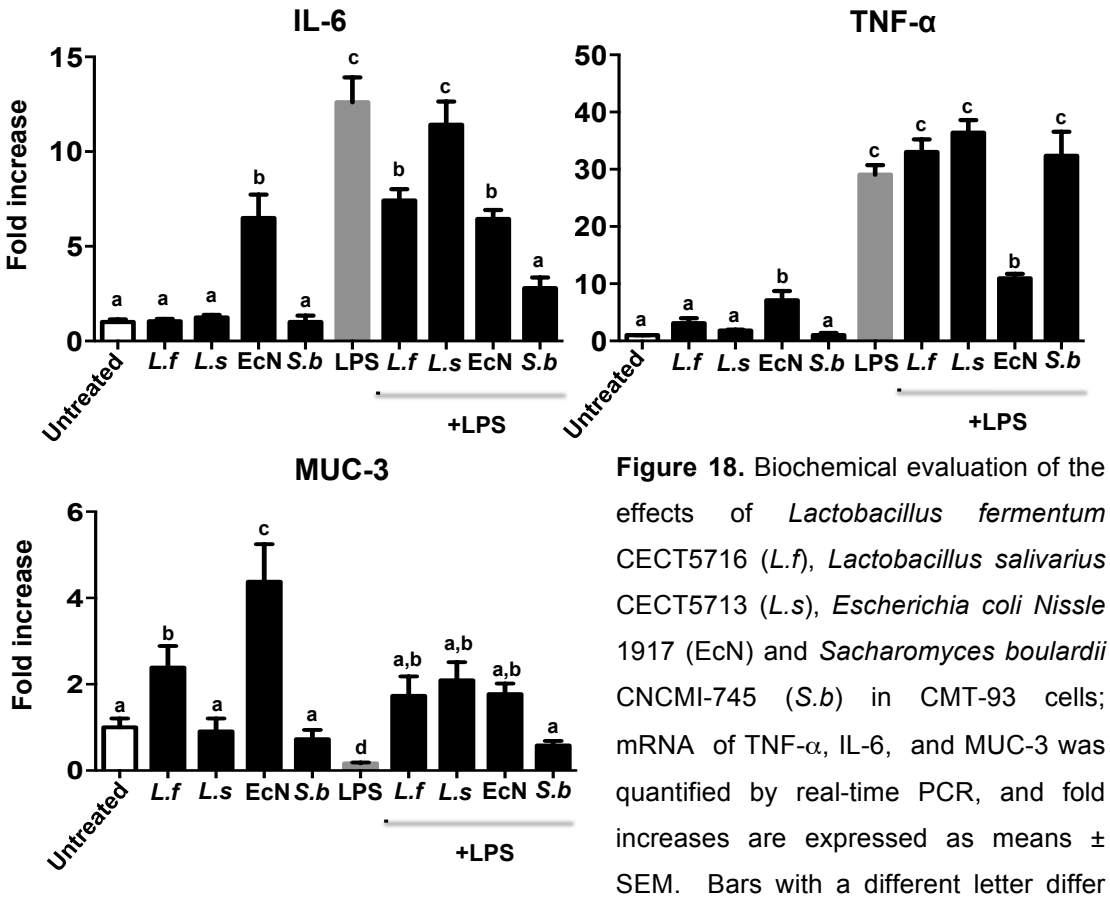


Figure 18. Biochemical evaluation of the effects of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*) in CMT-93 cells; mRNA of TNF-α, IL-6, and MUC-3 was quantified by real-time PCR, and fold increases are expressed as means ± SEM. Bars with a different letter differ statistically (P<0.05).

When the probiotics were incubated with BMDM without LPS-stimulation, the effects observed with the two lactobacilli assayed was different from that showed by *E. coli* Nissle 1917 and *S. boulardii*. Thus, both *L. fermentum* and *L. salivarius* were able to increase the expression of TNF-α, but only the latter upregulated the expression of IL-6, and no significant effect was observed with any of the lactobacilli when iNOS expression was considered. On the contrary, both *E. coli* Nissle 1917 and *S. boulardii* did increase the expression of the different markers tested in these macrophage cells (Figure 19).

The incorporation of LPS in the culture media of these cells increased the expression of the two cytokines and the inducible enzyme assayed, being the increased expression obtained higher than that achieved when the cells were incubated with the probiotics in basal conditions. In this experimental setting the incubation of the two lactobacilli with BMDM, before LPS-stimulation, resulted in the reduction of the increased expression of all the markers evaluated in the LPS-activated

cells, although *L. fermentum* showed a higher efficacy than *L. salivarius* (Figure 19). However, the incubation of *E. coli* Nissle 1917 and *S. boulardii* prior to LPS reduced the upregulated expression of the two cytokines assayed, but they did not show any significant modification when the expression of iNOS was considered (Figure 19).

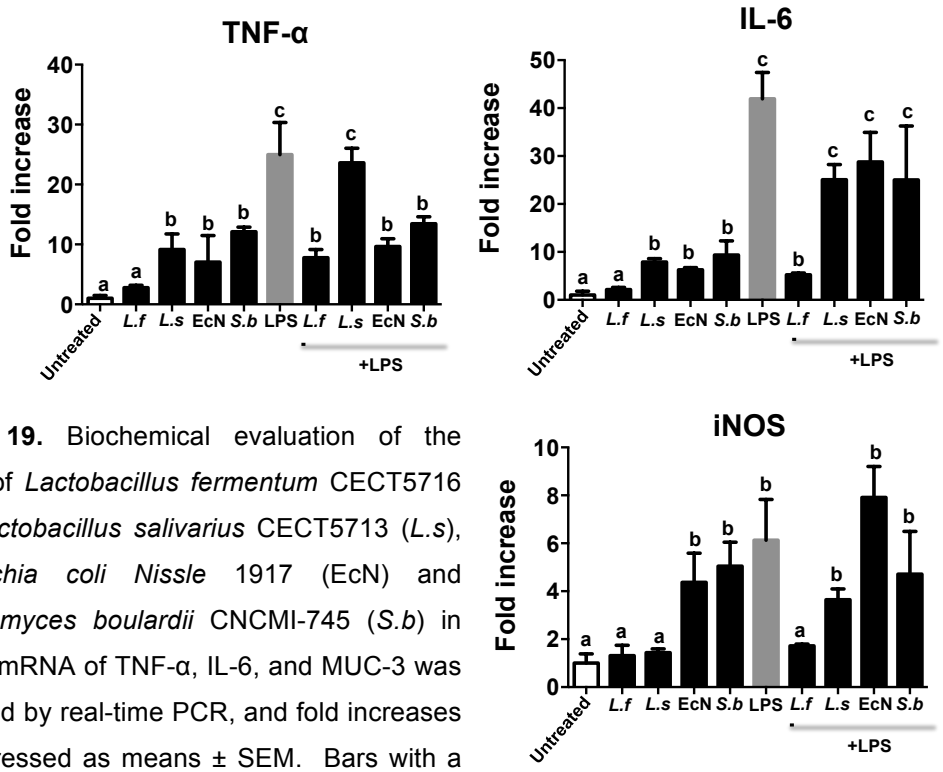


Figure 19. Biochemical evaluation of the effects of *Lactobacillus fermentum* CECT5716 (*L.f*), *Lactobacillus salivarius* CECT5713 (*L.s*), *Escherichia coli Nissle* 1917 (EcN) and *Sacharomyces boulardii* CNCMI-745 (*S.b*) in BMDM; mRNA of TNF-α, IL-6, and MUC-3 was quantified by real-time PCR, and fold increases are expressed as means ± SEM. Bars with a different letter differ statistically (P<0.05).

IMPACT OF THE PROBIOTIC VIABILITY ON THE INTESTINAL ANTIINFLAMMATORY EFFECT EXERTED BY LACTOBACILLUS FERMENTUM

1. Intestine anti-inflammatory effects of *Lactobacillus fermentum*, live or dead, in the TNBS model of rat colitis.

The administration of the probiotic *Lactobacillus fermentum*, live or dead, for 2 weeks before colitis induction did not result in any symptom of diarrhea or affect weight evolution in comparison with untreated rats (data not shown). However, the administration of the probiotic to colitic rats resulted in an overall lower impact of the TNBS-induced damage compared to the untreated colitic control group when

evaluated one week after the colonic insult. Thus, the intestinal anti-inflammatory effect was evidenced macroscopically by a significant reduction in the colonic damage score in comparison with that of control rats ($P<0.05$) (Figure 20) since a significant decrease of the area of colonic necrosis and/or inflammation was observed in both colitic groups treated with the probiotic. However, this anti-inflammatory effect was not associated with significant differences of the colonic weight/length ratio among colitic groups, which was increased significantly as a consequence of the inflammatory process (Figure 20).

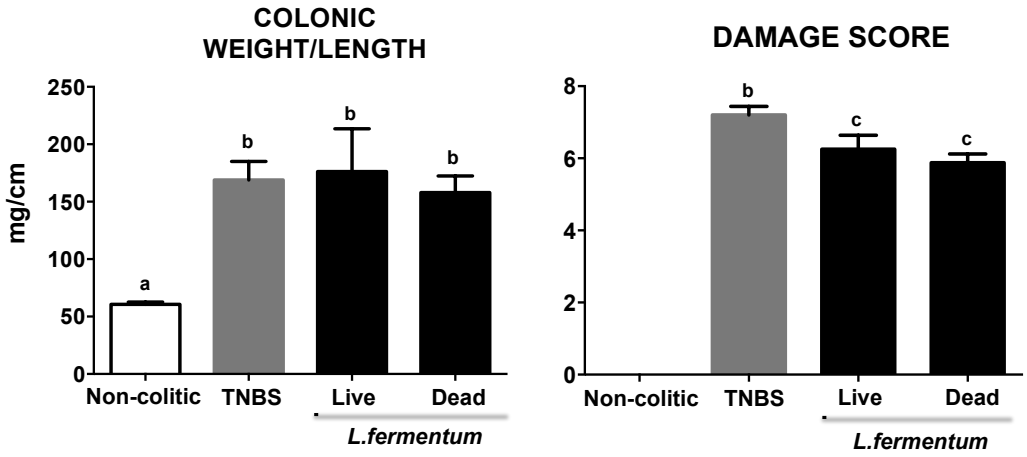


Figure 20. Effects of *Lactobacillus fermentum* CECT5716, live or dead, on clonic weight/length ratio and colonic macroscopic damage score. Bars with a different letter differ statistically ($P<0.05$).

Biochemically, the preventative beneficial effects showed by the probiotic, either live or dead, were evidenced by the reduction of the increased colonic MPO activity observed in the colitic control group (Figure 21), being this enzyme activity a marker of neutrophil infiltration (Krawisz et al., 1984). In addition, colonic inflammation was characterized by a decreased content in glutathione content, most probably as a consequence of the colonic oxidative stress induced by the inflammatory process, as previously reported in this model of experimental colitis (Peran et al., 2006) (Figure 21). The treatment with *L. fermentum*, live or dead, resulted in a significant increase in the colonic glutathione content, although no significant differences were observed between both treated groups (Figure 21).

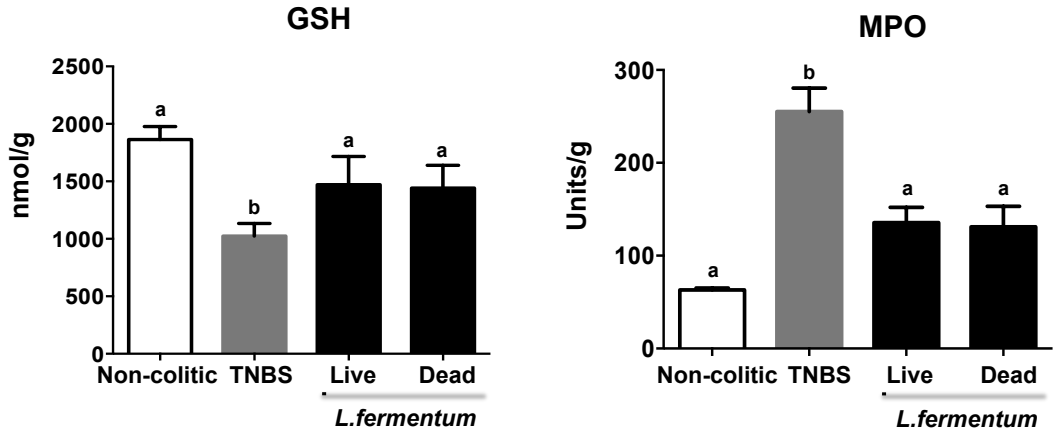


Figure 21. Effects of *Lactobacillus fermentum* CECT5716, live or dead, in TNBS rat colitis following a preventive treatment protocol. Colonic glutathione (GSH) content and colonic myeloperoxidase (MPO) activity. Bars with a different letter differ statistically ($P<0.05$).

Finally, the colonic inflammatory process induced by TNBS was also associated with increased levels of the pro-inflammatory cytokines TNF-α and IL-1β (Figure 22), as well as by a greater colonic iNOS expression (Figure 22) in comparison with non-colitic animals. The administration of the probiotic to colitic rats resulted in a significant reduction of both cytokine levels, and a lower colonic iNOS expression when compared to TNBS control animals, without showing differences between viable and death probiotic (Figure 22).

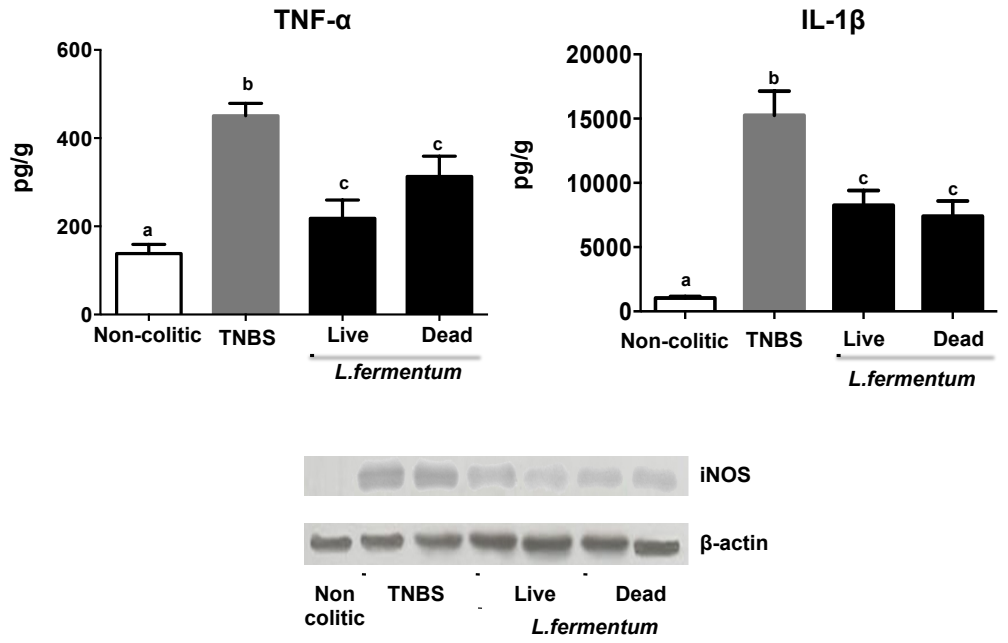


Figure 22. Effects of *Lactobacillus fermentum* CECT5716, live or dead, on colonic TNF-α and IL-1β production; and iNOS expression in TNBS rat colitis one week after damage induction. Data are expressed as means ± SEM; groups with a different letter differ statistically (P<0.05).

2. *Lactobacillus fermentum*, live or dead, inhibits the stimulated IL-8 production, p44/42 and p38 MAP kinase protein expression in Caco-2 cells.

To evaluate if the viability of *Lactobacillus fermentum* CECT5716 is essential to exert anti-inflammatory activity, we checked its immuno-modulatory effect in two in vitro models of cells involved in the intestinal immune response. The human colon adenocarcinoma cell line Caco-2 was used as a model of intestinal epithelial cells. The incubation of confluent Caco-2 cells with *L. fermentum* CECT5716, live or dead, for 3 h significantly increased the IL-8 production in comparison with basal conditions, without showing statistical significance when live or dead bacteria are considered (Figure 23.A). Also, incubation of these cells with IL-1β induces the secretion of IL-8, a pro-inflammatory chemokine that is released by intestinal epithelial cells and increases inflammatory cells migration from the blood stream into the mucosa and submucosa during chronic IBD, enhancing intestinal tissue destruction (MacDermott et al., 1998). In fact, the incorporation of IL-1β in the cell culture for 24 hours resulted in the stimulated release of IL-8, which was approximately 10-fold higher than that obtained

after the incubation of the cells with the probiotics on basal conditions (Figure 23.A). When Caco-2 cells were previously exposed to the probiotic, live or dead, a significant inhibition of the IL-1β-stimulated production of IL-8 was observed; however, in this case, the cell pre-treatment with live probiotic showed a higher reduction in this cytokine production than that with the dead bacteria (Figure 23.A).

The stimulatory effect of IL-1β on Caco-2 cells was associated with increased phosphorylation of the MAP kinases, both p42/44 ERK and p38 (Figure 23.B). The pre-treatment of these cells with live or death probiotic did not significantly modify the expression of these MAP kinases in basal conditions, but it showed inhibitory effects when they were stimulated with IL-1β, showing a reduced phosphorylation of the MAP kinase p42/44 ERK and p38 when compared with stimulated cells without probiotic (Figure 23.B).

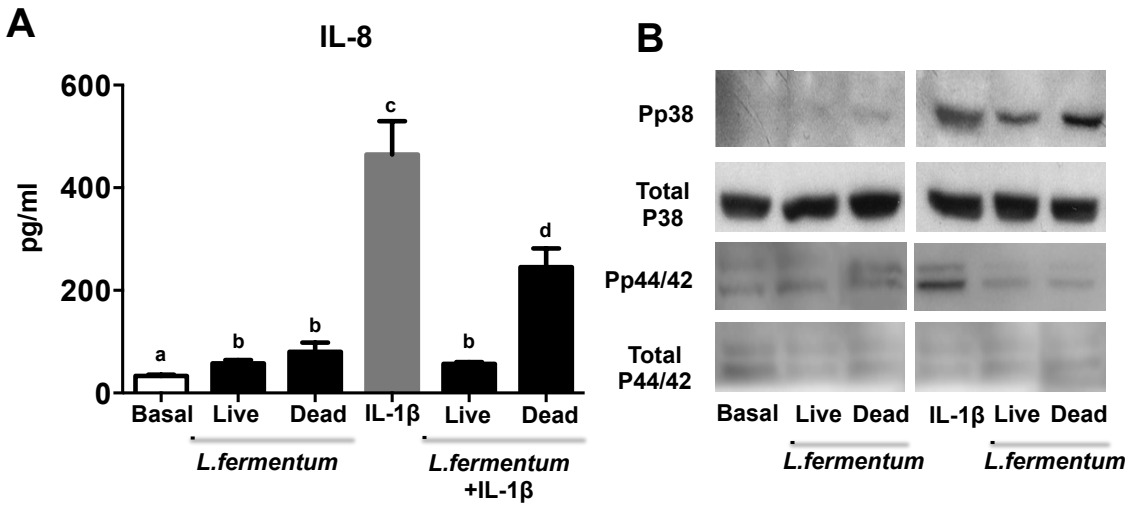


Figure 23. A. Effects of *Lactobacillus fermentum* CECT5716, live or dead, on IL-8 production in Caco-2 cells, in basal conditions and stimulated with IL-1β (1ng/ml). Data are expressed as means ± SEM. Bars with a different letter differ statistically ($P<0.05$). **B.** Effects of *Lactobacillus fermentum* CECT5716, live or dead, on p44/42 and p39 MAP kinase protein expression in Caco-2 cells in basal conditions and stimulated cells. The experiments were performed three times, with each individual treatment being run triplicate.

3. Lactobacillus fermentum, live or dead, inhibits nitric oxide and IL-1β production in stimulated RAW 264.7 cells.

Similarly to what occurred with Caco-2 cells, the incubation of confluent RAW 264.7 cells with *Lactobacillus fermentum*, live or dead, for 3 h resulted in a significant increase of the release of IL-1β and nitric oxide when compared with those cells without probiotic (Figure 24). LPS incorporation to the culture media of these macrophages for 24 hours resulted in an increased production of IL-1β and nitric oxide in comparison with the levels obtained in basal conditions, being these significantly reduced when the cells were previously incubated with the probiotic, either live or dead (Figure 24).

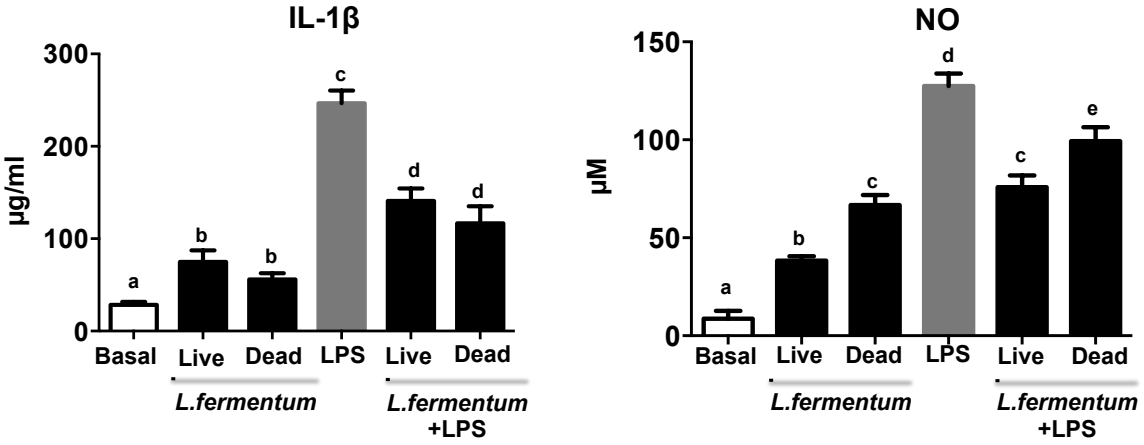


Figure 24. Effects of *Lactobacillus fermentum* CECT5716, live or dead, on IL-1β and nitrite production in RAW 264.7 cells, in basal conditions and stimulated with LPS (100 ng/ml). Data are expressed as means ± SEM. Bars with a different letter differ statistically ($P<0.05$).

Discussion

DISCUSSION

Intestinal antiinflammatory activity of selected probiotics: modulation of the immune response, impact on microbiota composition and role of micro-RNAs in experimental models of mouse colitis

The term IBD mainly comprises two gastrointestinal conditions, Crohn's disease (CD) and ulcerative colitis (UC). They are characterized by a chronic gastrointestinal inflammation disorder with alternating periods of relapses and remissions. The most common symptoms include diarrhoea, abdominal pain, blood in faeces, fever, nausea, anorexia, body weight loss, etc., which clearly lead to poor quality of life of the patients, and require prolonged medical and/or surgical interventions (Kaser et al., 2010). The incidence and prevalence of IBD is continuously increasing over the past decades in different regions around the world, especially in the most developing countries, causing an important socioeconomic burden (Molodecky et al., 2012). At present, the exact pathogenesis of IBD has not been fully elucidated; however, it is generally accepted that an altered function of the mucosal immune system takes place, which develops through a complex interaction between genetic factors, the host immune system and environmental factors. In this scenery, it is likely that the intestinal immune system triggers an exacerbated and uncontrolled response against unknown antigens located in the intestinal lumen (Hisamatsu et al., 2013). As a result, there is an up-regulation of the synthesis and release of different proinflammatory mediators, including reactive oxygen and nitrogen metabolites, eicosanoids, chemokines and cytokines, which actively contribute to the harmful cascade that initiates and perpetuates the inflammatory response in the gut (Strober & Fuss, 2011). Since a specific causal treatment for human IBD is not available yet, one of the main strategies to effectively downregulate the exacerbated immune response is to interfere with multiple stages of the inflammatory cascade, mainly by using aminosalicylates, glucocorticoids or immunosuppresants (Kho, Pool, Jansman, & Harting, 2001). More recently, biological drugs, like infliximab or adalimumab, which act by targeting TNF-α, a key early signaling molecule in the inflammatory cascade, have shown a higher efficacy in those patients who were resistant to conventional treatment (Randall et al., 2015). However, and unfortunately, these treatments are not devoid of potentially serious side effects, thus limiting their chronic use (Ngo et al., 2010; Stein & Hanauer, 2000). For this reason, it is interesting to develop new strategies that, in combination with safety, could restore the altered immune response

that emerges in the inflamed intestine in these conditions. When considering the latter, an imbalance on the intestinal microbiota, termed as dysbiosis, seems to play a key role (Abraham & Cho, 2009; Dalal & Chang, 2014). In consequence, an alternative in IBD therapy, rather than suppressing the immune system, could be to restore the composition of the altered microbiota, which can be achieved by the administration of antibiotics, prebiotics or probiotics (Sartor, 2004).

Several clinical trials have investigated the role of antibiotics in IBD patients and come up with conflicting results (Cammarota et al., 2015). But, despite the usefulness of antibiotics for the treatment of IBD, several studies have reported that discontinuation of antibiotic therapy results in a high relapse rate, and long-term antibiotic therapy is associated with increased risk of drug side effects and antibiotic resistance (Guslandi, 2005).

Prebiotics are defined as non-digestible, selectively fermented, ingredients that induce specific changes in the activity and composition of the gut microbiota, providing benefits upon host well-being and health (Roberfroid, 2007). Available data on the role of prebiotics in the management of IBD are actually few and fragmented, and most of the studies have been performed in experimental models of colitis (Cammarota et al., 2015). So, nowadays they cannot be considered as a real alternative to standard therapy in human IBD.

Probiotics have been defined by a joint Food and Agriculture Organization/World Health Organization expert consultation in 2001, as “live microorganisms, which when administered in adequate amounts confer a health benefit on the host” (Olier et al., 2012; Schultz, 2008). Specific bacterial strains have been suggested to play a protective activity against IBD, competing with pathogenic microbes or directly preventing their colonization in the gut, as well as through their anti-inflammatory properties (Kostic, Xavier, & Gevers, 2014). The available evidences do not conclusively support the use of probiotics in human IBD (Naidoo, Weaver, Stuart-Hill, & Tagg, 2011). Nevertheless, although practical guidelines are extremely cautious in recognizing the role of probiotics in these intestinal conditions and they do not advice the use of probiotics overall in the achievement of UC remission, they prudently recognize a reputed role for VSL#3 probiotc mixture. Moreover, among probiotics only *E. coli* Nissle 1917 has been proposed as an applicable alternative to mesalazine for the maintenance of remission of UC (Dignass et al., 2012). Similarly, ECCO-ESPGHAN pediatric guidelines do not support the use of probiotics for the induction nor for the maintenance of remission in UC, but they contemplate that probiotics, mainly VSL#3 mixture and *E. coli* Nissle 1917, may be considered with

caution in pediatric patients intolerant to mesalazine with mild active UC, or as an adjunct in children with mild residual activity despite conventional treatments (Turner et al., 2012). Of note, probiotics are "generally regarded as safe (GRAS)" by the U.S. FDA along with the World Health Organization (Mattia & Merker, 2008). This consideration about the safety of probiotics has been also extended to IBD patients (Veerappan, Betteridge, & Young, 2012), unless a situation of severe impairment of the gut barrier, with increased intestinal permeability, or a very compromised immune system function occurs, which have been considered as risk factors for the development of probiotic-dependent sepsis (Boyle, Robins-Browne, & Tang, 2006). When combining their potential benefits in intestinal inflammation and well-demonstrated safety, probiotics are interesting candidates to further study for their impact on intestinal microbiota and its role in IBD.

Additionally, since chronic inflammation in IBD is clearly associated with marked molecular changes in gene and protein expression (Xavier & Podolsky, 2007), small molecules involved in different cell pathways of these processes may be potential targets for IBD treatment. Among these, miRNAs are considered as promising candidates. They are a class of single-stranded non-coding RNA molecules on an average 22 nucleotides long, which are present in all eukaryotic cells except fungi (Bartel, 2004). miRNAs regulate gene expression both at a transcriptional and translational level, and mediate post-transcriptional gene silencing by directly binding to the 3' UTR of target mRNA (Candela et al., 2011). Several studies have reported the important role that miRNAs play in many biological processes, including signal transduction, cellular proliferation, differentiation, apoptosis and immune response (Bushati & Cohen, 2007; O'Connell et al., 2012). Moreover, miRNAs have been identified as crucial elements in the regulation of the innate and adaptive immune responses, and modifications in miRNAs expression have been related to different autoimmune diseases, like systemic lupus erythematosus, rheumatoid arthritis, psoriasis and IBD (Amarilyo & La Cava, 2012; Filkova et al., 2012; Pekow & Kwon, 2012; Schneider, 2012).

In the present study, and considering all the above, we have tried to get more knowledge about the mechanisms involved in the beneficial effects that probiotics may exert on intestinal inflammation. We have focused our analysis on the relationships among the modulation of the altered immune response exerted by probiotics, their impact on microbiota composition as well as the involvement of miRNAs in their activity, when they were assayed in two different models of experimental colitis in mice, induced by intracolonic administration of DNBS (Cannarile et al., 2009) or by

incorporation of DSS in the drinking water (Camuesco et al., 2012). Among others, these mouse models have been successfully used in the field of IBD to understand the pathogenic mechanisms of the disease as well as to perform the preclinical studies of potential drug treatments in humans (DeVoss & Diehl, 2014).

The probiotics used in the present Thesis were selected based on previous studies reporting their beneficial effects on intestinal inflammation, either in humans and/or in experimental models. These probiotics were *Lactobacillus fermentum* CECT5716 (Peran et al., 2006), *Lactobacillus salivarius* CECT5713 (Peran et al., 2005), *Escherichia coli* Nissle 1917 (Schultz, 2008; Arribas et al., 2009) and *Saccharomyces boulardii* CNCMI-74 (Guslandi et al., 2003; Garrido-Mesa et al., 2015).

The results revealed that all the probiotics assayed showed intestinal antiinflammatory effects in both experimental models of mouse colitis, thus confirming previous studies performed by our group as well as those reported by others (Damaskos & Kolios, 2008). However, the observed beneficial effect was dependent on the probiotic considered and not all of them displayed the same properties on the different markers of inflammation evaluated. Also, there were differences depending on the experimental model considered. In this sense, when macroscopically evaluated, the beneficial effects exerted by the probiotics were more evident in the DSS model than in the DNBS one. Thus, in the time-course evaluation, the DAI values obtained after DSS incorporation in the drinking water as well as afterwards, were lower in those groups of colitic mice treated with the probiotics than in the corresponding colitic control. The beneficial effects in the DSS model of experimental colitis were also evidenced in all probiotic-treated groups by a significant reduction in the colonic weight/length ratio, an index of colonic oedema that was increased significantly as a consequence of the inflammatory process (Camuesco et al., 2012). However, no beneficial effect was observed macroscopically in the DNBS-induced colitis after probiotic treatment, and no significant differences were obtained among colitic groups when body weight loss or colonic weight/length ratio were considered. The differences in the probiotic efficacy between both models may be due to the more intense colonic inflammatory response obtained after DNBS instillation.

However, the positive impact after probiotic treatment was demonstrated biochemically in both models, when the colonic expression of cytokines, chemokines, adhesion molecules or proteins involved in the intestinal epithelial integrity were evaluated by qPCR. Of note, these results evidenced the different intestinal antiinflammatory pattern showed by the different probiotics evaluated. All probiotics

reduced the expression of the pro-inflammatory cytokine IL-1β in both experimental models, whereas the expression of TNF-α was significantly downregulated by *L. fermentum* and *E. coli* Nissle 1917 only in the DNBS model. Furthermore, these two probiotics were also able to inhibit the increased expression of IL-12, when determined in DSS colitic animals. These results confirm the immunomodulatory properties ascribed to probiotics that account for their beneficial effects in intestinal inflammation (Stephani, Radulovic, & Niess, 2011). In fact, among the numerous cytokines, IL-1β, TNF-α and IL-12 have been considered as important inflammation mediators of innate and/or adaptive immunity, and to have a key role driving intestinal inflammation (Korzenik & Podolsky, 2006; Langrish et al., 2004). Typically, the activation of innate and adaptative immune responses during the progression of IBD also implies the increased expression of other mediators involved in the inflammatory response, like chemokines or adhesion molecules. Interestingly, probiotic treatments did not significantly modify the expression of MCP-1 or ICAM-1 in the DSS model of mouse colitis, excepting *E. coli* Nissle 1914, which was able to reduce the expression of ICAM-1 in colitic mice. This would mean that the beneficial effects observed with most of these probiotics would be unlikely related to the initial access of neutrophils and macrophages to the inflamed areas of the intestine. Instead, an inhibitory effect on the activity of immune cells could participate in the intestinal antiinflammatory effect. This was confirmed by the in vitro experiments performed with two immune cell types involved in the inflammatory response, intestinal epithelial cells (CMT93) and macrophages (BMDM). These assays revealed that the incubation of these cells with the different probiotics before LPS stimulation, which was used as an inductor of an inflammatory milieu, resulted in reduced expression of the proinflammatory cytokines IL-6 and TNF-α. Of note, when these probiotics were assayed in basal conditions, except *L. fermentum*, they were able to stimulate the expression of these cytokines in BMDM. Besides, only *E. coli* Nissle 1917 did increase their expression in the epithelial cells CMT-93. The ability of the probiotics to induced cytokine production, including TNF-α and IL-12, has been previously reported for *E. coli* Nissle 1917 and for other lactobacillus, *Lactobacillus casei* Shirota, in the murine monocyte/macrophage cell line J774A (Cross, Ganner, Teilab, & Fray, 2004). It therefore appears surprising that these probiotic strains are capable of down-regulating intestinal inflammatory responses in vivo, as demonstrated previously and in the present study, unless its mode of action is quite different when in contact with other immune cell types may promote the induction of antiinflammatory cytokines, like IL-10 or TGF-β, as it has been reported to occur with the probiotic *Lactobacillus paracasei* strain NCC2461 during an in vitro mixed lymphocyte reaction (von der Weid, Bulliard, & Schiffrin, 2001). Supporting this, the

intestinal antiinflammatory activity showed in the present study by the probiotics in the DSS model of colitis was associated with significantly increased expression of colonic TGF-β in comparison with colitic control group. TGF-β is a potent regulatory cytokine that inhibits Th cell proliferation, differentiation and activation, and decreases secretion of harmful cytokines (Wan & Flavell, 2007). It has been proposed that human IBD may be associated with defects in the counter-regulatory mechanisms against the excessive mucosal immune response that occurs in these intestinal conditions, such as those involving the immunosuppressive cytokine TGF-β. Indeed, studies in human IBD tissues and murine models of colitis have documented a disruption of TGF-β signalling (Monteleone, Caruso, & Pallone, 2012), and thus a restoration in its function can be a valuable strategy in these intestinal conditions (Marafini et al., 2013). In fact, the beneficial effects exerted by probiotics like *Bifidobacterium infantis* in TNBS-induced experimental colitis in mice have been attributed to its ability to increase Treg related molecules, including Foxp3, IL-10 and TGF-β (Zuo et al., 2014).

In addition to their effects related with the inhibition of the production of proinflammatory cytokines in activated immune cells, and probably with the increased production of antiinflammatory cytokines, the probiotics can affect the expression and/or activity of inducible enzymes involved in the inflammatory response. This may be the case of MMPs, a family of zinc and calcium-dependent proteolytic enzymes that degrade the structural proteins in the extracellular matrix, which have been also implicated in the pathogenesis of human IBD and experimental colitis (Baugh et al., 1999; Koelink et al., 2014). The results obtained in the present study revealed that, in DSS colitis, the probiotics reduced the expression of MMP-2 and MMP-9, excepting *E. coli* Nissle 1917, which did not reduce MMP-9. In fact, the inhibition of this enzyme expression has been associated with the intestinal antiinflammatory effects exerted by other drug treatments, like the tetracyclines minocycline and doxycycline, (Garrido-Mesa et al., 2011; Garrido-Mesa et al., 2015) and mesalamine (5-aminosalycilic acid) (Deng et al., 2009) in experimental colitis. During the last two decades, it has become increasingly clear that NO overproduction by iNOS is deleterious to intestinal function, thus contributing significantly to gastrointestinal immunopathology during the chronic inflammatory events that take place in IBD (Grisham, 2002). In the present study, iNOS expression was upregulated in both models of experimental intestinal inflammation used; however, only *L. fermentum* was able to significantly reduce the increased iNOS expression in colitic mice. It has been proposed that this probably results from the intense activation of macrophages, which took place as a consequence of the inflammatory insult (Camuesco et al., 2004). In fact, macrophages

are considered an important source of pro-inflammatory mediators, such as NO and TNF-α, playing a key role in the pathophysiology of IBD (Nielsen, 2014). Moreover, when the different probiotics were assayed in vitro in the macrophage cells BMDM, only *L. fermentum* was able to significantly reduce the LPS-upregulated expression of iNOS in these cells. This would suggest that the inhibition of iNOS activity could contribute to the intestinal antiinflammatory effect showed by this probiotic, and confirmed previous observations with the same probiotic in other experimental model of colitis, the TNBS model of rat colitis (Peran et al., 2007).

Finally, the impairment in the epithelial barrier function has been proposed to be one of the first events that occur in intestinal inflammation since it may facilitate the access of antigens from the intestinal lumen, triggering the exacerbated immune response (Mankertz & Schulzke, 2007; Su et al., 2009). Among the different factors that contribute to the maintenance of epithelial integrity, it has been also reported that colonic mucus layer plays a key role; in fact, human IBD has been associated with a defect in mucus production and a reduced number of goblet cells (Gersemann et al., 2009). These observations have been also reported in experimental models of rodent colitis (McGuckin et al., 2009), and confirmed in the present study, since the two models of colitis, DSS and DNBSS, were associated with a reduction in the expression of mucins MUC-2 (the primary constituent of the mucus layer in the colon), MUC-3 (a membrane-bound mucin), occludin (a transmembrane protein) and ZO-1 (an important linker protein in tight junctions that associates with occludin) which participate in maintaining the epithelial integrity (Furuse et al., 1994; Kim & Ho, 2010). The treatment of colitic mice with the different probiotics assayed significantly reversed the expression of the mucins MUC-2 and MUC-3, thus preserving the mucus-secreting layer that covers the epithelium and acts as a physical barrier protecting its integrity. Also, the expressions of occludin and ZO-1 were upregulated in those groups treated with the probiotics, and in consequence, these effects may contribute in facilitating the restoration in the epithelial barrier, thus promoting the recovery of the colonic damage. Although an indirect effect of the probiotics through the modulation of the altered immune response may be deleterious to the intestinal barrier function, a direct effect of these bacteria on epithelial cell cannot be ruled out in obtaining this beneficial effect. In fact, when the probiotics were assayed in vitro in the epithelial cell line CMT-93, all of them were able to counteract the reduced expression of the mucin MUC-3 induced after incubation of these cells with the inflammatory stimulus LPS. Moreover, both *L. fermentum* and *E. coli* Nissle 1917 upregulated the expression of this mucin even in basal conditions, which may produce preventative effects on epithelial integrity, which

has also been previously proposed for these probiotics to explain their intestinal antiinflammatory effects (Madsen et al., 2001; Ukena et al., 2007; Wu et al., 2008).

Considering all the above, we can conclude that the selected probiotics in the present study show intestinal antiinflammatory effects in two experimental models of mouse colitis, thus confirming previous observations obtained by our group as well as by others. Furthermore, and as it has been well reported, these beneficial effects can be associated with the well documented immunomodulatory properties of these beneficial bacteria and with their ability to preserve and facilitate the recovery of the intestinal barrier function.

Next, we wanted to know the impact of these probiotics on the microbiota composition in these experimental intestinal conditions, as well as if these beneficial effects were also associated with the modification of the expression of the miRNAs in intestinal inflammation.

As mentioned before, IBD is associated with an abnormal gut microbiota composition, called dysbiosis. However, the question that arises is whether the altered gut microbiota is a cause of disease or a consequence of the inflammatory state of the intestinal environment. At present, this question still remains unanswered. Therefore, as it was already commented in this Discussion section, the development of therapeutic strategies that combine an immunomodulatory activity and the ability to restore the luminal microbial balance in the intestine could be an interesting approach for the management of IBD. The administration of probiotics has been previously proposed to have the potential to reverse dysbiosis and restore tolerance towards the microbiota (DuPont & DuPont, 2011; Gareau, Sherman, & Walker, 2010; Sartor, 2004). However, several studies and meta-analyses of randomized trials performed with probiotics have reported that probiotics display varying success rates (Sazawal et al., 2006; Szajewska, Dziechciarz, & Mrukowicz, 2006). This can be due, at least partially, to the use of different probiotic strains that can elicit strain-dependent effects on the host.

In the last decade, the development of high-throughput 16S ribosomal RNA gene sequencing techniques has accelerated the knowledge of gut microbiome diversity (Hamady & Knight, 2009). Pyrosequencing allows the determination of the entire phylogenetic spectrum, taxonomic characterization, and provides the flexibility to analyze populations at different taxonomic levels, setting the stage for investigation of the IBD microbiome. It is known that the gastrointestinal microbiome of healthy humans is dominated by four major bacterial phyla: *Firmicutes*, *Bacteroidetes*, and to a

lesser degree *Proteobacteria* and *Actinobacteria* (Eckburg et al., 2005). Thus, an in order to gain insight into the functional consequences of IBD-associated dysbiosis, in the present study we used a novel approach pairing microbial community 16S gene sequence profiles with information from the closest available whole-genome sequences. Firstly, the results obtained by pyrosequencing were used to calculate 3 commonly used parameters to study the gut microbiota in DSS and DBNS models: Chao richness, Pielou evenness, and Shannon diversity (Chao, 2005; Colwell & Robert, 2009). Although all these 3 ecological parameters were significantly decreased in the DSS-colitic group, only Shannon diversity was found to be lower in the DNBS model. This discrepancy can be attributed to the differences in the pathogenesis of each animal model and may represent an important area of future research. Nevertheless, the reduced diversity showed in the present study has been also previously reported in IBD patients (Frank et al., 2007; Walker et al., 2011; Nagalingam & Lynch, 2012). When the probiotic treatments in colitic mice were evaluated, the results revealed a dependency on the probiotic used, since not all of them exerted the same modifications on the microbiota composition. Also, there were differences depending on the experimental model considered. In the DSS model, *L. fermentum* and *E. coli* Nissle 1917 were able to restore the 3 ecological parameters tested, whereas *L. salivarius* and *S. boulardii* did not showed significant modifications. By contrast, in the DNBS model of mouse colitis none of the probiotic treatments showed changes on Chao richness or Pielou evenness, although this can be due to the fact that these two parameters did not suffer any variation in this model. However, when the Shannon diversity was evaluated, the DNBS-colitic group showed a lower diversity value when compared with non-colitic mice, and this lower diversity was significantly increased after probiotic treatment to colitic mice with the two lactobacilli or with *E. coli* Nissle 1917, but not with S. boulardii.

In addition to the above parameters, another important marker, the F/B ratio, was also evaluated. This ratio was modified in both experimental models of colitis, although the modifications were not in the same way. Whereas in the DSS model the control colitic mice showed an increased F/B ratio compared with non-colitic group, in the DNBS model, colitic mice were characterized by a lower F/B ratio values. Similarly, these differences in the F/B ratio may be explained by substantial pathophysiological differences between both experimental models. The higher F/B ratio showed in DSS mice than in non-colitic group was associated with a higher abundance of *Firmicutes* and a fewer of *Bacteriodetes* (Figure 12.B). All the probiotic treatments restored this ratio except *S. boulardii*. This is consistent with our results when considering the data

obtained for the four principal phyla. Excluding *S. boulardii*, all probiotics reduced the abundance of the phylum Firmicutes compared with non-colitic group, although only the administration of *E. coli* Nissle 1917 was able to restore it without showing statistical differences with non-colitic mice. While some metagenomic studies have detected a reduced abundance of the bacterial phylum Firmicutes in experimental models of intestinal inflammation and in CD patients (Frank D, et al 2007; Atarashi K et al, 2011), others reports have revealed the key role that increased levels of *Clostridium difficile* can play in IBD patients and in experimental colitis (Kim et al., 2007; Issa et al., 2007). Moreover, it has been reported an increased abundance of *Ruminococcus gnavus*, also belonging to *Firmicutes* phylum, in IBD patients (Joossens et al., 2011; Sokol et al., 2009, Sokol et al., 2008). Therefore, the increase of the Firmicutes phylum observed in the present study in the DSS model could be related with the overgrowth of these subgroups of Firmicutes, thus increasing the value of F/B ratio in colitic mice.

Moreover, it is important to note the reduced abundance of *Bacteriodetes* observed in DSS mice versus the healthy group. In this case, all probiotic treatments were able to restore this abundance. These data agree with several studies that have shown the implication of *Bacteroidetes* in the normal development of the gastrointestinal tract. Gut *Bacteroidetes* generally produce butyrate, an end product of colonic fermentation which is thought to have ability to restore of energy metabolism in colonocytes, to reduce inflammation in experimental colitis due to its immunomodulatory activity, thus playing an important role in maintaining a healthy gut (Kim & Milner, 2007;Roediger, 1980; Böhmig et al., 1997).

Additionally, the results obtained in DNBS model showed a drastically reduction of the F/B ratio in colitic mice. This reduction was associated with a lower quantity of *Firmicutes* without modifying *Bacteroidetes* levels in comparison with non-colitic group. Once again, only *L. fermentum* and *E. coli* Nissle 1917 administration significantly increased this ratio value when compared with untreated colitic mice, although the results in the mean abundance only reflect a tendency in restoring the reduction in *Firmicutes* species. Similar shifts in this bacterial composition have been reported by others investigators in IBD patients (Gophna et al., 2006; Sokol et al., 2009; Baumgart et al., 2007; Andoh et al., 2009).

It is interesting to note that the observed discrepancies among the different studies may be in part explained by different disease phenotypes, environmental differences and the complexity of the sequencing performed in each study. Additionally, individual bacterial species present unique pathological effects and, similarly, shifts in gut bacterial colonies can also prompt specific disease-inducing

activity (dysbiosis) or disease-protective activity (probiosis). Considering all the above, at present the findings have suggested that in order to further advance our understanding of health and disease in IBD, we will require a better characterization of the variability in the microbiota, a better understanding of how such variability can result in similar or different functional profiles, and more integrative studies that take into account the interaction between the microbiota, the host, and the environment to produce a phenotype.

Finally, the study was focused on the role of miRNAs in the intestinal antiinflammatory effect of the probiotics. As it has been indicated before in this Thesis, miRNAs are small, endogenous noncoding RNAs that post-transcriptionally regulate gene expression. They are generated from long primary transcripts with the participation of different protein complexes. Thus, the RNAse-III-type enzyme Drosha first processes them in the nucleus; then, miRNA precursor is exported to the cytoplasm, where the final cleavage is performed by Dicer, aRNAse III like enzyme. This enzymatic process yields RNA duplexes of about 21 nucleotides length with typical ends (5 phosphates and 2 nucleotides 3' overhangs) (Meister &Tuschl, 2004). In June 2014, approximately 36,000 mature miRNAs occurring across all species had been registered in the miRbase (http://mirbase.org). miRNAs are involved in several biological processes, including development, cell differentiation, proliferation and apoptosis; furthermore, it is estimated that miRNAs may be responsible for regulating the expression of nearly one-third of the genes in the human genome (Griffiths-Jones et al., 2006). Thus, miRNA deregulation often results in an impaired cellular function, and a disturbance of downstream gene regulation and signaling cascades, suggesting their implication in disease etiology (Kloosterman & Plasterk, 2006). This explains the great effort made by the scientific community during the last decade for studying the impact of miRNA on human diseases and for their treatment as well as diagnosis. Actually, their participation has been documented in almost all pathologies, including those affecting the cardiovascular system, metabolic ailments, neurological disorders, infectious diseases, as well as in cancer and inflammation, including human IBD (Pfeifer & Lehmann, 2010; Arcanioti et al., 2011).

Although the modification in the pattern expression of miRNAs has been also previously reported in different experimental models of colitis, we first decided to characterize the expression of some of the miRNAs in the two experimental models we have used in the present Thesis, according to our experimental settings. With this aim, we initially developed a customized array of 80 different selected miRNAs, according to an inflammatory panel, in the colonic tissue from mice submitted to DSS or DNBS

colitis and compared with non-colitic mice. In these preliminary assays, approximately eleven miRNAs showed modifications in their expression, but only three of them in the DSS model (miR-155, miR-223 and miR375) (Figure 25.A) and four in the DNBS model (miR150, miR-155, miR143 and miR-223) (Figure 25.B) reached statistical differences in comparison with non-colitic mice group. Based on these results, we selected these five miRNAs to be evaluated in the in vivo assays performed to evaluate the intestinal antiinflammatory effect of the probiotics.

miR-150 and miR-155 have been reported to be involved in the regulation of the immune response, and the control of the development and function of innate immune cells, but also respond to proinflammatory/anti-inflammatory signals, including pro-inflammatory cytokines, and influence the release of cytokines and chemokines as well (Chen et al., 2004; O'Connell et al., 2010). Consequently, their expression has been also described to be altered in intestinal inflammation. Thus, miR-150 was significantly increased in the epithelial cells of colonic mucosa in UC patients compared with controls, and suggested an inverse correlation between miR-150 and its target, c-Myb, a proto-oncogene involved in apoptosis (Bian et al., 2011; Xiao et al., 2007). Moreover, miR-150 was found to be elevated in mouse colon treated with DSS compared with the control colon (Bian et al., 2011). This has been confirmed in the present study in the DSS model, but not with de DNBS model, in which a reduced expression of this miRNA was obtained in control colitic mice in comparison with the healthy group. These discrepancies in the expression of miR-150 in intestinal inflammation have been reported both in humans and in experimental models. In this sense, Bao et al., (2014) have described that miR-150 was found significantly downregulated in human colitis as well as in *MUC2* KO mice of colitis-associated cancer model, associated with the corresponding upregulation of different pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6. Similarly to what occurred with miR-143, both *L. fermentum* and *E. coli* Nissle 1917 were able to restore the colonic expression of miR-150 in both experimental models, that is, to reduce its expression in DSS colitis and to upregulate it in the DNBS model. However, *L. salivarius* and *S. boulardii* showed the same effect in both experimental models, which was to increase its expression, especially when the yeast was considered, since 15-fold increase expression was obtained. Similarly, miR-155 expression has been reported to be upregulated in the colonic mucosa of IBD patients (Fasseu et al., 2010; Takagi et al., 2010), thus supporting the data obtained in the present study, since an increased expression was obtained in colitic control mice in both experimental models of colitis. This miRNA seems to play a key role in the intestinal inflammatory process,

since miR-155-deficient mice have been reported to be resistant to DSS-induced colitis (Singh et al., 2014), In fact, miR-155 has been identified and characterized as a component of the primary macrophage response to different types of inflammatory mediators (O'Connell et al., 2007). In the immune system, upregulated miR-155 expression plays an important role in the differentiation of B and T cells (Faraoni et al., 2009), driving the response of activated dendritic cells (Martinez-Nunez et al., 2009), and contributes to the development of Th17 and regulatory T cells (Kohlhaas et al., 2009; Singh et al., 2014). It is relevant that all the probiotics were able to significantly reduce the expression of miR-155 in colitic mice, thus supporting that they show immunomodulatory properties that can account for their beneficial effects in these experimental models of colitis.

The miRNA miR-223 has been described to have a key role in the development and homeostasis of the immune system, mainly in monocyte/macrophage differentiation. Thus, it is repressed when granulocyte–monocyte progenitors start to differentiate into monocytes and it is highly expressed when granulocyte–monocyte progenitors enter the granulocyte differentiation phase (Johnnidis et al., 2008). Furthermore, this miRNA is also expressed CD4+ T helper lymphocytes when activated in different diseases like rheumatoid arthritis (Fulci et al., 2010). To date, miR-223 has been involved in many types of cancers, inflammatory diseases, autoimmune diseases, among other pathological processes (Taïbi et al., 2014). It can be considered that miR-223 acts as an inflammatory miRNA, by regulating the production of the inflammosome complex, closely related with IL-1β production in immune cells (Haneklaus et al., 2012). When the expression of this miRNA was evaluated in the experimental models of colitis used on the present study, an upregulated expression was obtained in both models, and similarly to what happens with miR-155, all probiotic treatments were able to restore it to normal levels compared to healthy mice. As commented above, miR-223 is involved with IL-1β production in different immune cells. It is interesting to note that, in the present study, we have checked that the inhibition in the expression of miR-223 obtained with the different probiotics correlates with their effects when the colonic expression of IL-1β was evaluated.

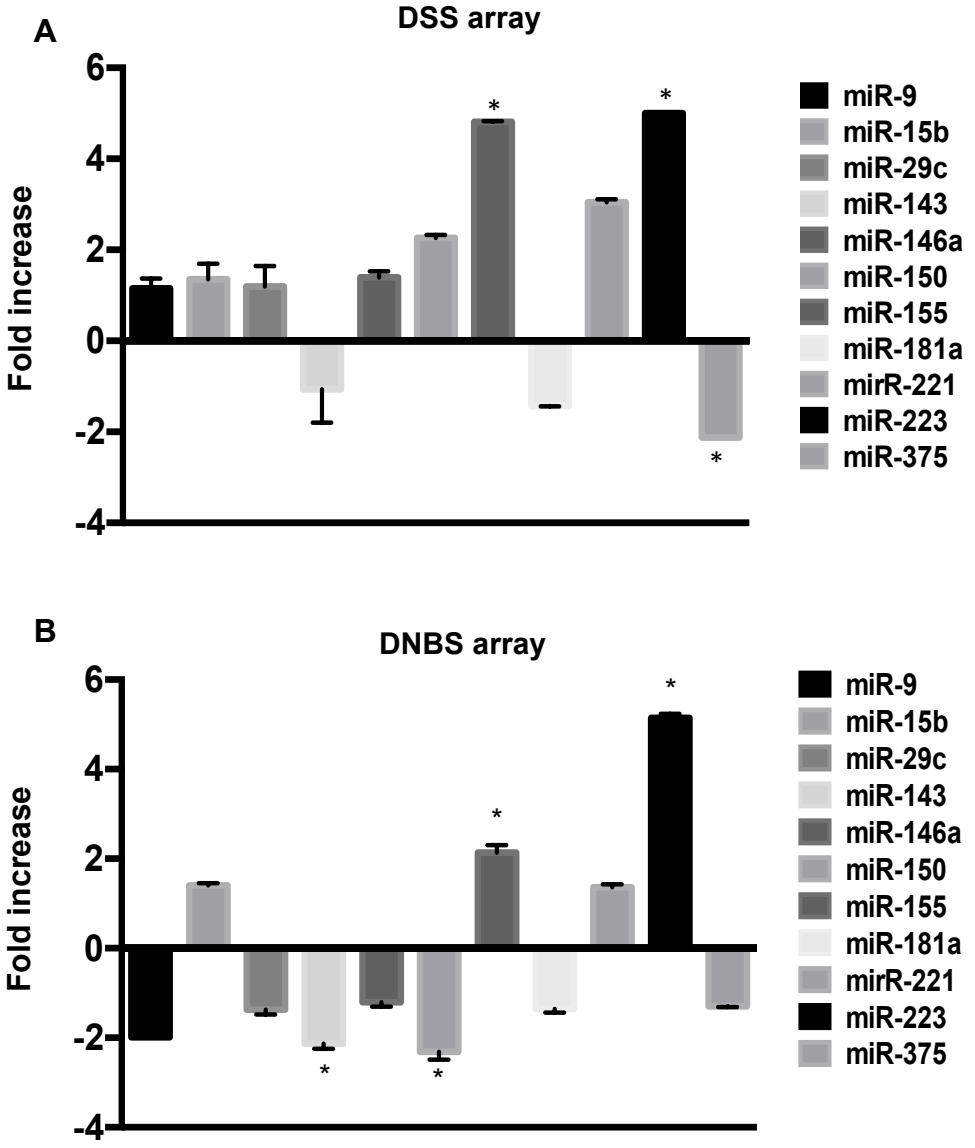


Figure 25. miRNA PCR array. **A.** Expression of different miRNAs in DSS model. **B.** Expression of different miRNAs in DNBS model.

Although all the probiotic showed mostly a similar effect when the above miRNAs are considered, it is evident that additional and characteristic mechanisms must be involved for every probiotic that could justify the differences observed in the efficacy among them as intestinal antiinflammatory agents.

miR-143 is highly expressed in the colonic tissue and seems to play important roles in normal colonic biology, most probably related with controlling cell turnover. In fact, it has been described that its loss contributes to neoplastic progression, since it is downregulated early in colon cancer (Michael et al., 2003; Akao et al., 2006). Moreover, it has been also found that loss of miR-143 induces pro-inflammatory signals in the innate immune system (Starczynowski et al., 2010). Since epithelial cell

turnover is increased in UC and UC-associated dysplasia is believed to increase the risk of malignant transformation (Shinozaki et al., 2000). Pekow et al., (2012) evaluated the expression of miR-143 in patients withlongstanding ulcerative colitis. They conclude in this study that this miRNA was significantly downregulated in these patients, whereas their predicted targets, insulin receptor substrate 1 (IRS-1), Kirsten rat sarcoma viral oncogene homolog (K-RAS), apoptosis inhibitor 5 (API5), and MEK-2 were up-regulated. They also postulated that loss of this tumor suppressor miRNA would predispose to chronic inflammation and neoplastic progression in IBD. Experimental models of colitis-associated cancer, like the azoxymethane (AOM)-DSS model, have confirmed these observations (Josse et al., 2014). Thus, chronic DSS treatment, either in mice without AOM administration or AOM-pretreated, downregulated miR-143 expression, being this associated with activation of the PI3K/Akt pathway, which may, therefore, represent molecular links between inflammation and cancer (Davis et al., 2014). The possible role of miR-143 in intestinal inflammation has been also confirmed in the present Thesis, since its expression was significantly downregulated in the two experimental models of colitis used, DSS and DNBS. When the probiotic treatments were evaluated, it is interesting to note that only *L. fermentum* and *E. coli* Nissle 1917 were able to restore the colonic expression of miR-143 in both experimental models, and thus preservation of the function of this miRNA on intestinal cells may contribute in the intestinal antiinflammatory properties observed with these probiotics. Of note, it has been reported that miR-143 also plays a crucial role in the vascular system, since it participates, together with other miRNAs, such as miR-145, miR-1 or miR-24, in the differentiation and proliferation of vascular smooth muscle cells (Wei et al., 2013). Several studies have shown that miR-143 is downregulated in injured vessels (Cordes et al., 2009). Studies performed in collaboration with the Cardiovascular group in our Department of Pharmarmalogy have demonstrated that an altered vascular function occurs in experimental intestinal inflammation (unpublished results), and thus, a restoration on these miR-143 levels, and a possible amelioration in the altered vascular function may also contribute to the beneficial effects showed by these probiotics.

Finally, miR-375 is known as a multifunctional miRNA involved in pancreatic islet development, glucose homeostasis, cell differentiation and carcinogenesis (Xu et al., 2011). Different studies have revealed controversial results regarding the modification of its expression depending on the diseases considering that both downregulation and upregulation have been reported to occur (Ding et al., 2010; Zhao et al., 2012). Besides, the modification in its expression is not constant in IBD, and it

may be used as a marker to identify and discriminate between CD and UC, because its expression was altered in a statistically significant manner in CD, but not in UC (Schaefer et al., 2015). In experimental colitis, it has been reported that miR-375 expression was significantly lower in IL-10$^{-/-}$ mice when the pathology was developed compared to normal mice (Schaefer et al., 2011). Similarly, the results obtained in the present study revealed that miR-375 expression was significantly downregulated in both experimental models. However, only *L. salivarius* was able to partially and significantly counteract this decreased expression in colitic mice, which could contribute to the beneficial effects showed by this probiotic.

In conclusion, the beneficial effects showed by these probiotics are associated with their ability to modify the immune response, which can be achieved at a post-transcriptional level by modifying miRNAs expression. It is important to note that only a few studies have been reported to date in which a correlation between the beneficial effects of probiotics and the modulation on the expression of miRNAs has been done. In fact, only two in vitro studies can be considered closely related to our study. One of these studies described the ability of inactivated strains of two lactobacilli, LGG and *L. delbrueckii subsp. bulgaricus*, to affect TLR4 expression in human monocyte-derived DCs, being these effects associated with the modifications in the expression of miR-146a and miR-155 (Giahi et al., 2012). In another study, Veltman et al. (2012) showed that *E. coli* Nissle 1917 differentially regulated the expression of different miRNAs, miR-203, miR-483-3p, miR-595, which are involved in the regulation of the barrier function by modulating the expression of regulatory and structural protein components of tight junctional complexes.

The intestinal anti-inflammatory properties of non-viable *Lactobacillus fermentum* CECT5716

As commented before, the probiotics could exert their effects through different mechanisms, and some of them could be attributed to the interaction of probiotics with other microorganisms, either members of the microbiota or potential pathogens, which result in the restoration of the dysbiosis that characterizes these intestinal conditions (Gerritsen, Smidt, Rijkers, & de Vos, 2011). It is evident that this type of interaction is typically dependent on the viability of probiotics. In addition, other mechanisms are related to the cross-talk between probiotics and host cells, clearly contributing to the well-known immunomodulatory properties ascribed to these beneficial bacteria. However, and in contrast to the direct effects exerted by the probiotics on the microbiota composition, their interaction with the host cells is not exclusively

dependent on the bacterial viability, due to the capacity of immune cells to recognize specific bacterial components or products, thus promoting the corresponding immunological response (Adams, 2010).

In fact, the in vitro assays performed in the present study confirm this possibility, since in both intestinal epithelial cells and macrophages, the probiotic viability does not appear to be essential to affect cell activity, both in basal conditions and when the cells are stimulated with IL-1β or LPS, respectively. Furthermore, the cellular mechanisms involved in these effects seem to be similar, because when the expression of the MAP kinases p42/44 ERK and p38 were evaluated in unstimulated or stimulated epithelial cells, both live or dead probiotic showed the same pattern of activities. It is well known that cell-wall components from Gram-negative such as lipopolysaccarides as well as host-derived cytokines such as IL-1β and TNF-α, increase IL-8 secretion from intestinal epithelial cells through the activation of MAPK (Jijon et al., 2012; Otte, Cario, & Podolsky, 2004). In consequence, the ability of the probiotic to modulate MAPK activity can justify its effects on IL-8 production in intestinal epithelial cells.

These results confirmed that this strain of *L. fermentum* exhibits one the important features of potential probiotic candidates; that is, the capacity to modulate the immune response of the host, which clearly contributes to its intestinal anti-inflammatory effect, as stated in the in vivo experiments performed in the present study. However, it is interesting to note that these beneficial effects are not exclusively dependent on the probiotic viability, since both live and dead probiotic ameliorated colonic inflammation induced by the instillation of TNBS in rats.

Classically, the pathogenesis of IBD was mainly attributed to an exacerbated adaptive immune response against antigens present in the luminal environment of the intestine. Most recently, a novel hypothesis has proposed that this inflammatory disease of the gut may result from a primary defect in intestinal innate immunity, which in turn could cause an imbalance between immune responses and tolerance to the gut microbiota that leads to chronic intestinal inflammation (Bamias, Corridoni, Pizarro, & Cominelli, 2012). Considering this, the immune modulatory properties of the probiotic *L. fermentum*, as evidenced both in vitro and in vivo in the present study, seem to play a key role. Thus, in normal conditions, i.e. when the intestinal mucosa is not submitted to any offending agent, the probiotic can promote the strengthening in the immunological barrier by stimulating and maintaining the state of alert of the innate and adaptive immune system. In fact, the in vitro assays showed an increased production of the innate cytokines IL-8 and IL-1β, as well as of NO, when either

intestinal epithelial cells or macrophages were incubated with probiotic. A similar overproduction of cytokines has been reported for PBMCs exposed to well-established probiotic strains of lactobacilli, streptococci, *Leuconostoc* spp., and *Bifidobacterium breve* (Gaudana, Dhanani, & Bagchi, 2010; Kekkonen et al., 2008).

However, in an inflammatory environment, the probiotic is able to decrease the exacerbated immune response, as confirmed both in vitro and in vivo. In vitro, the probiotic inhibited the stimulated production of IL-8 (intestinal epithelial cells), IL-1β and NO (macrophages). In vivo, probiotic treatment to colitic rats resulted in reduced colonic production of the pro-inflammatory cytokines TNF-α and IL-1β, as well as a downregulation of colonic iNOS expression, when compared to the untreated corresponding controls. IL-8 is a chemokine that stimulates migration of neutrophils from intravascular to interstitial sites and can directly activate neutrophils and regulate the expression of neutrophil adhesion molecules (Huber, Kunkel, Todd, & Weiss, 1991). In consequence, the ability of this probiotic to decrease IL-8 production can contribute to inhibit leukocyte infiltration in the inflamed tissue in colitic rats, as evidenced by the lower colonic MPO activity values observed in treated colitic rats in comparison with the untreated colitic group. An increase in neutrophils is a key feature in the pathogenesis of colitis in humans and animals (Williams et al., 2000), which once activated by different stimuli, including IFN-γ, promots the release of reactive oxygen species products, such as hydrogen peroxide and hypochlorous acid, leading to a situation of oxidative stress and causing local tissue damage (Steinbeck, Roth, & Kaeberle, 1986). This has been corroborated in the present study by a depletion of colonic glutathione content, which was partially prevented after probiotic treatment. In fact, reducing or limiting the influx of these proinflammatory cells has been previously demonstrated to attenuate inflammation (Farooq et al., 2009), and this could be one of the mechanisms involved in the beneficial effect showed by this strain of *L. fermentum*. Similarly, different studies have also reported the ability of other probiotics, like *Enterococcus faecalis*, to modulate and attenuate the inflammatory responses to further prevent inflammatory diseases, such as necrotizing enterocolitis in infants, through interaction with the expression and production of IL-8, as a result of MAPK signaling pathway inhibition (Wang, Hibberd, Pettersson, & Lee, 2014). Besides, in the DSS-colitis model, similarly to what occurs in patients with IBD, p38 levels are increased in the colonic tissue (Ihara et al., 2009), and when treated with p38 inhibitor, mucosal IL-1β and TNF-α levels were reduced in DSS colitis model (Hollenbach et al., 2004), consistent with what has been found for *L. fermentum* treatment in the present study.

Conclusions

CONCLUSIONS

1. Probiotics, administered as a preventative treatment, display an intestinal anti-inflammatory effect in both experimental model of acute colitis induced by DSS and DNBS in mice. Their ability to modify the intestinal microbiota composition and the immunomodulatory properties that characterize these bacteria are involved in the beneficial effects. Regarding the latter, the probiotic treatments show a positive impact on the innate immune response, preserving the intestinal barrier integrity and decreasing the production of pro-inflammatory cytokines, and on the adaptive immune response, modifying the expression of Th1-, Th17- and Treg-related cytokines. In addition, these actions can be achieved at a post-transcriptional level by modifying the expression of some miRNAs, which is altered as a consequence of the intestinal inflammatory status.

2. All probiotics evaluated, similarly to other probiotics used in inflammatory bowel disease therapy, exert a direct immunomodulatory effect, evidenced in vitro in colonic epithelial cells and macrophages, in which they restored the expression of different markers implicated in the immune response.

3. The mechanisms involved in the anti-inflammatory effects seem to be dependent on the particular probiotic, since each one showed its own pattern of modulating the expression of the different markers evaluated as well as modifying the intestinal microbiota composition.

4. The viability of the probiotic *L. fermentum* CECT5716 is not required for its anti-inflammatory activity. Both conditions live and dead, have demonstrated to attenuate the inflammatory process in the TNBS model of rat colitis as well as in vitro. This would provide an additional value to the probiotic when orally administered to obtain a beneficial effect on the host, and may imply the revision of the generally accepted probiotic concept, since the viability is considered as a requisite to exert its beneficial effects.

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Abbreviations

ABBREVIATIONS

5-FU	5-fluorouracil
AI-2	Second autoinducer
AIEC	Adherent-invasive *Escherichia coli*
AMP	Antimicrobial peptide
AOM	Azoxymethane
APC	Antigen presenting cell
API5	Apoptosis inhibitor 5
ATG16L1	Autophagy-related protein 16-1
BMDM	Bone marrow derived macrophage
CAM	Adhesion molecule
CARD15	Caspase recruitment domain 15
CCL20	Chemokine (C-C motif) ligand 20
CD	Crohn's disease
CFU	Colony forming units
Chr	Chromosome
CLA	Conjugated linoleic acid
COX-2	Cyclo-oxygenase-2
DAI	Disease activity index
DC	Dendritic cell
DLG5	Disks large homolog 5 gene
DMEM	Dulbecco's modified Eagle's medium
DNA	Desoxirribonucleic acid
DNBS	Dinitrobenzene sulphonic acid
DSS	Dextran sodium sulphate
EcN	*Escherichia coli* Nissle 1917
EHEC	Enterohemorrhagic *Escherichia coli*
ELISA	Enzyme-Linked ImmunoSorbent Assay
emPCR	Emulsion-based clonal amplification
ER	Endoplasmic-reticulum
FBS	Fetal Bovine Serum
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRAS	Generally regarded as safe
GSH	Total glutathione

GWAS	Genome-wide association studies
Hath1	Atonal homolog 1
hBD-2	Human ß-defensin 2
Hes1	Hairy and enhancer of split-1
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICAM	Intercellular adhesion molecule
IEC	Intestinal epithelial cells
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL10R	Interleukin 10 receptor
IL18RAP	Interleukin 18 receptor accessory protein
IL23R	Interleukin 23 receptor Intestinal epithelial cells
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
IRGM	Immnuty-related GTPase family M
IRS-1	Insulin receptor substrate 1
JAK	Janus kinase
K-RAS	Kirsten rat sarcoma viral oncogene homolog
KC	Keratinocyte chemoattractant
KLF4	Kruppel-like factor 4
KO	Knockout
L.f	*Lactobacillus fermentum* CECT5716
L.s	*Lactobacillus salivarius* ssp. *salivarius* CECT5713
LAB	Lactic acid bacteria
LFA	Lymphocyte function- associated antigen
LGG	*Lactobacillus rhamnosus* GG
LincRNA	Large intervening non-coding RNA
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
M-CSF	Macrophage colony-stimulating factor
Mac-1	Macrophage 1 antigen
Map	Mucouss adhesion-promoting protein
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein

MDP	Myeloid differentiation primary response protein
MHC-II	Major histocompatibility complex-II
MIP	Macrophage inflammatory protein
miRNA/miR	Micro-RNA
MMP	Metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger RNA
MUB	Mucus-binding protein
Myd88	Myeloid differentiation primary response protein
NF-κB	Nuclear factor-kappa B
NK	Natural killer cell
NKT	Natural killer T cells
NLR	NOD-like receptor
NLRP	NOD-like receptor pyrin
NO	Nitric oxide
NOD2	Nucleotide oligomeration domain 2
OCLN	Occludin
OCTN1	Organic cation transporter 1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKB/Akt	Anti-apoptotic protein kinase B
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
RDP	Ribosomal data project
RhoB	Ras homolog family member B
RIPK2	Receptor-interacting serine-threonine kinase-2
RNA	Ribonucleic acid
RORγt	Retinoic-acid-receptor-related orphan receptor-γt
ROS	Reactive oxygen species
S.b	*Saccharomyces boulardii* CNCMI-745
SCFA	Short-chain fatty acids
SCID	Severe combined immunodeficient

SHH	NOD2-sonic hedgehog
SIBO	Small intestinal bacterial overgrowth
SNORD95	Small nucleolar RNA, C/D box 95
SNP	Non-coding single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor β
Th	T helper cell
TIR	Toll-IL-1 receptor
TJ	Tight junction
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulphonic acid
TNF	Tumour necrosis factor
TNFSF	Tumour necrosis factor ligand superfamily
TRAF	Tumor necrosis factor receptor-associated factor
Treg	T regulatory cells
TSB	Tryptone soya broth
TYK	Tyrosine kinase
UC	Ulcerative colitis
UTR	Untranslated region
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VLA	Very late antigen
ZO	Zonula occludens

Anexo

Mi formación predoctoral dentro del grupo de investigación "Farmacología de productos naturales" me ha permitido participar en diferentes proyectos, cuyo trabajos han sido publicados en prestigiosas revistas científicas a nivel internacional. Asimismo, esta formación se ha visto complementada con la realización de cursos, asistencia a congresos, nacionales e internacionales, y estancias en otros centros.

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CURSOS Y OTROS MÉRITOS

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AGRADECIMIENTOS

Es difícil entender la importancia de los agradecimientos de una tesis doctoral hasta que no se ha terminado. En ese momento te das cuenta de cuánto tienes que agradecer a tanta gente. Dice el refranero popular que “es de bien nacido ser agradecido”. Por lo tanto, me gustaría en este apartado agradecer a todas aquellas personas que de una u otra forma, me han ayudado a realizar este trabajo, sin ellas no hubiera sido posible. Hoy todo dejar de ser un sueño para pasar a ser una realidad.

En primer lugar, quiero expresar mi más sincero agradecimiento a mis tres directores por haberme ayudado y dirigido durante todo este tiempo. Al Dr. Julio Juan Gálvez Peralta, por toda tu ayuda y sabios consejos. ¡Gracias Julio! Agradezco que me hayas abierto las puertas del grupo, dándome la oportunidad de tener una visión más amplia del mundo de la investigación y descubrir cuánto me motiva. Tus comentarios y análisis de lo que iba escribiendo siempre han sido acertados, críticos, cariñosos,… geniales como sólo tú sabes serlo. Agradecerte toda la confianza depositada en mí, por transmitirnos tu filosofía de trabajo, haciéndonos ver que el buen trabajo se hace en grupo; aunque parezca anecdótico, gracias también, por todos los agobios y presiones, así he comprobado que todo es posible con trabajo y esfuerzo; gracias por convertirnos en una gran familia; por tu ánimo y apoyo en todo momento; por ser tan cercano y amable; por ser gran docente y aún mejor persona. A la Dra. Maria Elena Rodríguez Cabezas que siempre ha estado ahí para lo que he necesitado, directora y a la vez, compañera y amiga. Y por último, al Dr. Carlo Riccardi, que me abrió las puertas de su departamento y me hizo sentirme en casa estando tan lejos de la mía.*Grazie mille a lui e il suo gruppo*.

Gracias al Dr. Antonio Zarzuelo, por todo el interés mostrado durante mi trabajo. Agradecerle su apoyo y su confianza. Gracias por tener siempre una sonrisa y palabras de ánimo. Aunque no tuve el lujo de ser tu alumna, sé que fuiste un gran profesor y ha sido un orgullo haberte conocido. Gracias por todo.

A todo el grupo de Biosearch S.A., por hacer posible la realización de esta Tesis Doctoral. En especial, a la Dra. Mónica Olivares y al Dr. Óscar Bañuelos.

Gracias a la Dra. Pilar Utrilla, compañera, amiga y una bellísima persona. He aprendido mucho de ti tanto a nivel personal como profesional. Auténtica, coherente, clara y bueno….“Maravillosa”. Siempre con palabras de ánimo y sensatez, has hecho que mi paso por aquí sea mucho mas sencillo.

Gracias a la Dra. Mónica Comalada, dispuesta a orientarme en el mundo de la ciencia siempre que lo he necesitado, a pesar de la distancia.

Gracias a mis compañeros, ¡cuánto habéis aguantado! A mis *"blancaflores"*, Frá, Marta y Teresa gracias por todo. No tengo palabras para poder expresar todo lo que siento por vosotras. Frá, gracias por ser mi compañera y amiga siempre, a pesar de todo; gracias por aguantar todos mis momentos, incluidos los días de bipolaridad, gracias por tener siempre algo bueno que decirme, gracias por estar ahí de forma incondicional. Hemos aprendido juntas y hemos estado a veces en momentos "chungos" pero siempre nos hemos complementado. Muchas cosas te debo, gracias por ser como eres. Ha sido un lujo poder compartir contigo estos años de trabajo y locura. Eres una *"monstrua".* El sol que brilla todos los días, mi Martuki, contigo rompieron moldes, generosa, buena persona, amable, cariñosa, sincera, disponible siempre a todo, a pesar de que no tengas tiempo nunca para nada, empática y un sin fin de adjetivos positivos! Gracias a Teresa "Cioci"….gracias por tu sonrisa diaria, por tu saber escuchar y aconsejar, por hacerme entender que los problemas lo son, si uno le da importancia, por ser la "alegría de la huerta", por tu disponibilidad a ayudar en todo momento; y por toda tu generosidad. A Pepe, por tu ayuda, por todo el interés que has mostrado en el trabajo, por tu curiosidad científica que sé que te llevará lejos... Gracias Dra. Natividad Garrido Mesa, Nati, mil gracias por todo, siempre dispuesta, una "todo-terreno", una pena no haber compartido más tiempo junto a ti…. Gracias a tod@s por vuestro cariño, habéis sido unos grandes compañeros y mejores amigos.

Gracias al grupo de "Cardio". Gracias al Dr. Juan Manuel Duarte, gracias por tu cariño y simpatía. Agradecerte siempre tus palabras de apoyo, ser tan cercano y hacer mas afable el trabajo en la facultad. Gracias al Dr. Miguel Romero, "Miguelito", por ser como eres, agradable, risueño, aunque a veces con "mala pipa" se te quiere. Me has ayudado mucho cuando estaba perdida en Farmacia y siempre he podido contar contigo para enseñarme, apoyarme y cómo no echar unas risas. A la Dra. Rosario Jiménez Moleón, Chari, que siempre ha estado ahí para lo que he necesitado, ofreciéndome su ayuda en todo momento. Al Dr. Manolo Sánchez, poco hemos coincidido pero suficiente para darme cuenta de la gran persona que eres. Muchas gracias por toda tu ayuda, por ese humor irónico que tuve el placer de descubrir en Glasgow y por todo lo que me has ayudado; que no ha sido poco. A la nuevas incorporaciones, Iñaki y María, que han sido aire fresco al grupo y al departamento, gracias por vuestro interés y vuestras palabras de ánimo.

A todos y cada uno de los miembros de este departamento, por vuestra amabilidad y por todos los momentos que he podido compartir con ellos durante estos

años. Al Dr. Severiano Risco, Seve, gracias por ser tan auténtico, encontrarte ha sido un verdadero placer. Gracias a ti y a tu mujer, la Dra. Cristina Mediavilla, fue lógico encontrarme con una gran mujer….. Ha sido un placer haber trabajado y aprendido con los dos. Gracias por toda vuestra ayuda. A Pedro, "Peter", con el que aprendí al inicio de esta aventura y echamos muy buenos ratos, te agradezco esa buena acogida, gracias. A Belén Rivero, por nuestra complicidad desde el principio, por toda tu ayuda, siempre con una sonrisa, por nuestras "charlitas", por hacerme sentir una más en *farma.* A Borja, gracias por tus palabras y por todo el apoyo en este último empujón. A Mercedes González, por su paciencia con pedidos imposibles; y por toda su ayuda. A Andrea, Cristina e Isa por su simpatía y amabilidad en todo momento.

A la Dra. Eva Valdivia Martínez y el Dr. Manuel Martínez Bueno, del Departamento de Microbiología de la Facultad de Ciencias de la Universidad de Granada. Agradecerles la oportunidad que me dieron, con vosotros empezó todo.

Al Dr. Federico García y la Dra. Natalia Chueca del Servicio de Microbiología del Hospital San Cecilio de Granada, por abrirme las puertas del laboratorio y poder usar todo lo necesario para la realización de mi trabajo. Gracias por enseñarme y ayudarme, a pesar de llegar con conocimientos muy pero que muy básicos he aprendido muchísimo a vuestro lado.

Agradecer a todo el que ha pasado por nuestro laboratorio, con los que hemos disfrutado, aprendido y nos han enriquecido a muchos niveles. Gracias Cinthia, Lynda, Nike, Oyin, Fatima, Smain, Claudio, Nourdine, Izaskun, Samah, Asia, Alex, Daline, Yasmim, Martina, Roberta, ….Gracias a tod@s.

Thanks to Dra. Stolarczyk, who very gently, without knowing me, accepted to help me. Thank you for everything.

A *Jesu* por aparecer en mi vida... ¡No te imaginas cuánto! Dicen que las penas son menos penas en compañía y si esa compañía es la tuya, ni penas son. Por todas esas horas de trabajo, yo en mi ordenador, de espaldas sin hablarnos, pero juntos. Por esos: ¿te importa leer esto que he escrito?, por esos otros: ¿me revisas la ortografía de este apartado?, por aquellos: estoy pensando que…. ¿qué te parece?… en definitiva por aguantar mis defectos, manías, peticiones, enfados, etc. Gracias por tu interés, tu amor, por aguantarme en todo momento, por estar ahí siempre, aunque, en alguna ocasión no te lo haya puesto fácil. Mil gracias por tu ayuda, y sobre todo, por ofrecérmela siempre. Gracias por escucharme, por preocuparte por mí, por entenderme, por cuidarme.

A mis amigos, que han sabido disculpar mis ausencias y siempre han tenido una palabra de ánimo. Estoy absolutamente convencida de que si de algo puedo presumir en esta vida es de los grandes amigos que tengo, lo que me hace sentir una

persona muy afortunada. No hace falta que los nombre, ellos saben quienes son y lo importantes que son para mi y, aunque algunos están lejos, tengo la suerte de poder sentirme siempre acompañada.

A mi familia, por ese soporte incondicional que siempre me han dado para poder hacer lo que me ilusionaba sin cuestionarme. A todos mis ti@s y prim@s, que siempre han tenido palabras de apoyo y por los que me siento muy orgullosa. Gracias, a mi tia "Loly" por ser como eres, por haberme inculcado el amor por la cultura y por quererme tanto. Gracias a mis abuelas, orgullosas de mí, siempre, lo entendieran o no. Gracias por todo, por vuestro esfuerzo, por vuestra manera de entender la vida en la que la honestidad y solidaridad han sido claves. Gracias también, a aquellas personas que han estado presentes en mi camino y, aunque ya no están, han puesto su granito de arena para que hoy sea quién y cómo soy.

¿Y cómo terminar sin el reconocimiento a los primeros brazos y a los primeros hombros en los que uno se sube en la vida? A mis padres, quienes en ocasiones han dejado de lado sus propios sueños e intereses para que cumpliera los míos. Sin mis padres y mi hermano, sin su incondicionalidad, aliento y estímulo nunca habría sido capaz de alcanzar esta meta… Sin duda, sin su gran apoyo y compañía este gran sueño no habría sido lo mismo. Gracias por haber sido capaces de mirar más allá, por animarme a continuar con mi formación y por ofrecerme siempre todo lo mejor. Gracias, por haberme enseñado que la vida es para los valientes.

…….Mi agradecimiento va para todos vosotros……Por fin he llegado al principio……